CANCER RESEARCH

VOLUME 11 AUGUST 1951 NUMBER 8

Symposium
Intermediary Carbohydrate Metabolism in Tumor Tissue

(The following four papers by V. R. Potter, R. E. Olson, S. Weinhouse, and P. C. Zamecnik were presented in Cleveland, Ohio, on April 29, 1951, before the 42nd Annual Meeting of the American Association for Cancer Research, Inc.)

Studies on the Reactions of the Krebs Citric Acid Cycle in Tumor, with Homogenates, Slices, and *in Vivo* Technics*†

VAN R. POTTER

(McArdle Memorial Laboratory, Medical School, University of Wisconsin, Madison 6, Wis.)

As a biochemist I am honored to be asked to participate in a symposium at a meeting of this association, and I am sure that my colleagues on the program are as grateful as I am for an opportunity to present one segment of the biochemical point of view for your consideration. We do not advocate this view to the exclusion of others. Instead, we feel that in the presentation of our views to this audience we can benefit by hearing the comments and criticisms of our fellow-members of the association who are more experienced in the intricacies of cancer biology than we are.

In the time allotted to me I should like first to discuss the broad aspects of the problem, in order to show why the study of oxidative carbohydrate metabolism of tumors may be relevant to the phenomena of their uncontrolled growth. Following this, I would like to trace the development of my own thoughts on the Krebs cycle oxidations in tumors in terms of the experiments with which we have been engaged. As recently as a year ago there was considerable uncertainty as to the status of the Krebs cycle in tumor tissue. However, during the past year, advances in the laboratories of all of the speakers on today's symposium have done much to dispel this uncertainty, while at the same time raising new issues. It seems reasonable to hope that this symposium may help to illuminate the new problems that we face by clarifying the position which we have now reached.

Turning now to the general aspects of today's symposium, we might first attempt to justify the study of carbohydrate oxidation in tumors. In order to do this we have to consider growth from the biochemical point of view. In growing cells there is a continual synthesis of protein, nucleic acids, and other complex substances. Nearly all of these large and complicated molecules are built up from smaller molecules. It is important to note that the smaller building blocks do not necessarily have to be built into new cell material. Instead, they may have many alternative pathways open to them, as was recently emphasized in a review by Potter and Heidelberger (16). Thus, a molecule of lactic acid, for example, does not have to be

* Figures and tables presented in the symposium are not reproduced here if bibliographic citations are available.

† This work was supported in part by grants from the American Cancer Society as recommended by the Committee on Growth of the National Research Council.

burned to carbon dioxide and water. Under certain conditions it may be converted along an *alternative* pathway through several intermediate steps and combined with ammonia to form glutamic acid, which as an amino acid can be used to build protein. These alternative pathways are like switches in a railroad yard, and the fact of their existence raises the question of whether the *increased* growth of tumor tissues can be related to a *decrease* in the oxidative pathways operating to switch the flow into a pathway resulting in growth. This seems at first like a simple and naïve way to explain the complicated phenomenon of uncontrolled growth, but we have shown in one case that the actual operation of such a metabolic switch can be a very subtle and intricate mechanism (23). It is an underlying assumption in this work that by starting

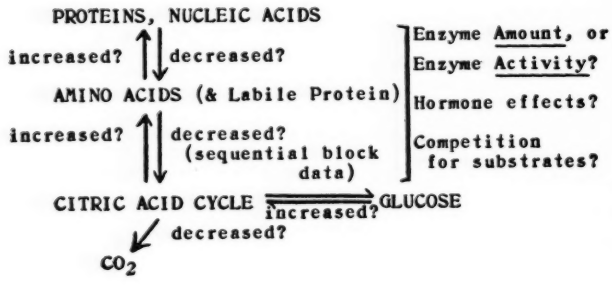


CHART 1.—Possible explanations for continuing protein and nucleic acid synthesis in tumor cells.

with known metabolic pathways such as the Krebs citric acid cycle we can work back to switches or branches as yet unknown, and come finally to one or more switches of far-reaching importance. As Zamecnik (27) recently said, "The interrelations of protein, carbohydrate, lipid, and nucleic acid chemistry are being found to be increasingly numerous, and an alteration in the metabolism of one may likely produce an effect in all." No doubt this is a view in which all of the speakers concur. There are hundreds of anastomoses and interconnections in the metabolism of all of these substances, and each of us enters the maze in terms of his own background.

The immediate problem of the present symposium is the nature of oxidative carbohydrate metabolism in tumors, but this problem must be viewed in relation to other metabolic pathways and to the larger problem of *growth control*. It is important to note that the Krebs citric acid cycle is significant not only for carbohydrate oxidation but also because it forms the terminal mechanism by which fats and proteins are ultimately oxidized to carbon dioxide. This is an additional basis for entering the maze at this point, although entry at *any* point quickly involves the whole complex of reactions.

The present symposium probably will not involve anaerobic carbohydrate metabolism. The rapid conversion of glucose to lactic acid in tumors is now quite generally understood to occur by way of the phosphorylated intermediates of the Embden-Meyerhof scheme, owing to the excellent work of LePage, of Meyerhof, and others. New studies on the alternative pathways that may lead to the synthesis of the ribose and desoxyribose sugar components of nucleic acids and nucleotides are in progress in several laboratories and may reach a decisive stage within the next few years.

For the present, however, we will be more concerned with oxidative carbohydrate metabolism and its possible relationship to growth. For some reason, as yet unknown, the tumor acts as a "nitrogen trap," to use a term coined by Mider and coworkers (8). By this it is meant that instead of having the nitrogen output approximately equal to the input, as in the normal nongrowing cell, the tumor cell takes in compounds containing nitrogen and uses them to synthesize protein and nucleic acid for continued cell duplication. Of course, certain normal cells are able to do this too, but the process is somehow controlled.

If we look at the process in more detail, in terms of the metabolic switches or alternative pathways referred to earlier, we can set up several categories or over-all stages in metabolism and begin to test the various possibilities. As we examine Chart 1, we can see that the increased activity of the synthetic processes in tumors could conceivably come about either as an increase per se in one pathway, or by a decrease in an opposing or competitive process involving the same compound. When we begin to talk about "increases" or "decreases," we have to ask what shall be the standard for comparison, what control tissues are most appropriate, and what kind of information our technic will yield. Some of the answers to these questions may be brought out by following the course of my own search for controls and for technics.

Before describing the work carried out in our laboratory, it should be mentioned that the idea of a deficiency of oxidative enzymes in tumor tissue may be found in a number of earlier studies. Time does not permit an adequate survey of the early work of Warburg (25), the perfusion experiments of the Coris (3, 4), the work on cytochrome c by Euler and others (see 5), on succinoxidase by Elliott and by Salter (see 24), and the demonstration of high levels of lactic acid in tumors *in situ* by LePage (7). Some of these studies, in retrospect, raise the question of the rate of blood flow through

tumors and the rate of oxygen consumption *in situ* based on A-V differences for which data do not appear to be available.

I should now like to mention briefly some of our own studies on the amount of various oxidation enzymes in tumor tissues. Emphasis was placed upon quantitative estimations in so far as possible rather than a report of presence or absence. In the early work we were greatly aided by Dr. DuBois and Dr. Schneider, who were students at that time. We began by measuring the cytochrome c content of the various tissues (5) and progressed to cytochrome oxidase and succinic dehydrogenase (24) and later to the malic dehydrogenase system (13). More recently, a cytochrome reductase has been studied by Dr. Reif (22). In the earlier studies, from five to ten specimens of each tissue were tested. A pattern began to emerge, according to which certain tissues were high in these closely related enzymes. These tissues were heart, kidney, liver, and brain, given in decreasing order. Another group of tissues contained much less of these enzymes, and contained amounts similar to those found in the various kinds of tumors. These studies were greatly illuminated by a number of studies made at St. Louis by Carruthers and Suntzeff, one of which (2) is included for comparison with our figures (Table 1). This emphasizes the problem of controls, for if liver tumors are compared with liver they are low in succinoxidase (24), but if skin tumors are compared with skin they are high in succinoxidase (2). These results may indicate that the *ratio* between different kinds of enzymes may be more significant than individual values.

TABLE 1

SUCCINOXIDASE ASSAYS OF VARIOUS NORMAL AND TUMOR TISSUES (REFERENCES 2, 24)

Tissue	Succinoxidase Q_{O_2}
Rat heart	219
" kidney	195
" liver	88
" brain	49
" spleen, lung, ten different types of rat and mouse cancers including hepatomas and embryonic rat liver and brain	10–25
Mouse skin cancer	16
Mouse skin (epithelium)	4

In 1945 Lehninger demonstrated fatty acid oxidation in the absence of cell structure in preparations from liver, and we were able to confirm his observations using whole isotonic homogenates, although at first we were misled by a technic that measured disruption of mitochondria instead of whole cells (12, 19). In a subsequent study we were unable to detect any fatty acid oxidation in hepatoma homogenates under the same conditions that were effective with liver (17). This finding can now be easily explained in the light of subsequent results to be mentioned below.

By 1945 we were also studying the whole Krebs cycle with its coupled phosphorylations and oxidative systems. In this system there are so many components that the limiting enzyme is not clearly defined, and maximum oxygen uptake depends upon the presence of a number of substrates. We chose oxalacetic acid, because its removal could be followed analytically and because it would provide pyruvate (19). The latter fact meant that with one substrate the Krebs cycle could be initiated. We now know that a mixture of pyruvate and fumarate is probably better for this purpose. The Krebs cycle and fatty acid oxidations in whole homogenates or preparations derived therefrom are remarkable in that the relation between the oxidations and the process of phosphorylation is still very intimate, just as it is in the intact cell. However, there are two features that are noteworthy. First, the rate of oxidation is governed by the rate of ATP breakdown and, second, the maintenance of the oxidative enzymes is dependent upon the maintenance of ATP (Chart 2). In an intact tissue or in a tissue slice, the intracellular enzymes are segregated, and phosphate breakdown is regulated. When we homogenize these cells, enzymes formerly segregated are dispersed, and phosphate breakdown increases. In tissues that have a high level of Krebs cycle enzymes, this increased phosphate breakdown results in a rate of oxygen uptake in homogenates that is much greater than can be attained in a slice. These tissues are heart, kidney, liver, and brain. However, in tissues in which phosphate breakdown cannot be counterbalanced

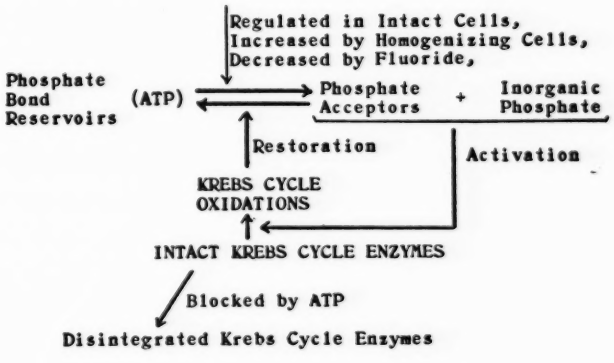


CHART 2.—Breakdown of adenosine triphosphate in relation to activation and maintenance of Krebs cycle oxidations.

by the Krebs cycle oxidations, the oxidative enzymes quickly disintegrate in homogenates. The latter situation is found in the case of tumor tissues.

In 1948 (19), we reported that there was no detectable oxidation of oxalacetic acid in various tumor homogenates. It was pointed out at that time that excessive phosphate breakdown might explain the results,[1] and subsequent studies on tumor homogenates proved this to be the case (20). However, at that time we attempted a study in which the phosphate reservoir was maintained by glycolysis, but found no evidence for the oxidative removal of oxalacetate (18), possibly because oxalacetate is a powerful hydrogen acceptor.

At about this time, Dr. Heidelberger set up facilities at the McArdle Laboratory for work with radioactive isotopes, and it was possible to test for the oxidation in tissue slices, in which excessive phosphate breakdown and enzyme loss might not occur. Labeled acetate was added to tissue slices, and the $C^{14}O_2$ was collected and counted. The rate in the tumor slices was about 3 per cent as great as in kidney, and the data were in line with the earlier reports by Elliott *et al.* (6), in which acid disappearance was low in tumors when pyruvate was the substrate. These acetate studies by Pardee, Heidelberger, and Potter (10) strengthened the growing conviction that a key enzyme was lacking in the Krebs cycle in tumor tissues. The idea was further strengthened by a study by Potter and Busch (14) on citrate formation in the tissues of animals in which citrate oxidation could be blocked by injecting fluoroacetate as shown by Buffa and Peters (1). No citrate accumulated in tumor tissues. However, a report by Olson and Stare claimed (9) that labeled *pyruvate* could be oxidized by tumor slices. Both carbonyl- and carboxyl-labeled pyruvate were prepared, and a new series of slice experiments was undertaken with the help of Mr. Watson. The data confirmed both our earlier work in that *acetate* was very poorly oxidized, and the work of Olson and Stare in that *pyruvate* was effectively oxidized (Potter, Watson, and Heidelberger).[2] Without going into details, it was found that Flexner-Jobling carcinoma slices oxidized carbonyl-labeled pyruvate to CO_2 at a rate that was about 25 per cent as high as that of kidney. The use of inhibitors of stages in the Krebs cycle were in line with the idea that the pathway proceeded via the Krebs cycle. However, when acetate was used, an entirely different picture was obtained. In confirmation of earlier results (10) the Flexner-Jobling tumor failed to oxidize acetate, and, moreover, fluoroacetate was without effect. Acetate was oxidized to only $\frac{1}{100}$ the extent seen in kidney. The addition of glucose to improve the viability of the tumor slices had a significant but slight effect. When hepatoma slices were used, the acetate oxidation was about $\frac{1}{10}$ as great as that of kidney. Meanwhile, Dr. Weinhouse had obtained acetate oxidation as well as fatty acid oxidation in tumor slices and had kindly sent us his data (26). At present no explanation of the difference in results is available. Our data were obtained with only one to two slices of tumor or kidney per flask, and there was no bicarbonate or CO_2 present. Dr. Weinhouse used larger amounts of tissue, and this may possibly explain the difference.

In the light of the slice results, i.e., pyruvate oxidation in tumor slices, we returned to the idea that the phosphate breakdown in tumor homogenates was excessive and had to be blocked. Fluoride was the logical addition, and at fairly high levels of fluoride the homogenate (Flexner-Jobling carcinoma) was able to oxidize pyruvate and fumarate, at rates equivalent to that in slices (20). Earlier tests with fluoride added to kidney and liver had given inhibition. Now, of course, it is obvious that fluoride will *inhibit* if phosphate breakdown is *limiting* and will be of *benefit* if phosphate breakdown is *excessive:* Since the rate of oxidation is dependent upon the availability of both inorganic phosphate and a phosphate acceptor, presumably adenosine diphosphate (ADP), and since fluoride inhibits the breakdown of ATP to ADP and inorganic phosphate, the addition of fluoride to liver and kidney homogenates will inhibit the rate of oxidation, because in these tissues the Krebs cycle enzymes can convert ADP to ATP faster than phosphatase action can dephosphorylate ATP. However, since too much ATP breakdown is accompanied by the breakdown of one or more components of the enzyme complex responsible for the Krebs cycle oxidations, the inhibition of this breakdown will have a beneficial

[1] "The observation . . . that isotonic tumor homogenates are unable to oxidize oxalacetate under the conditions described above does not prove that the original tumors are devoid of one or more of the enzymes required for this oxidation, although it seems likely that the tumors must contain very low amounts of the enzyme system. The data could also be obtained if the phosphorylating mechanisms that maintain the oxidative system (and are maintained by it) are inadequate to keep pace with the decay of the oxidative system: the isotonic tumor homogenates that have been studied could have little or none of the oxidative system or they could have a preponderance of the breakdown mechanisms such that no activity could be demonstrated under the conditions employed. In either case, the fact remains that a marked difference between a number of normal tissues and tumors has been demonstrated" (19).

[2] V. R. Potter, L. S. Watson, and C. Heidelberger, The Oxidation of Labeled Pyruvate and Acetate in Tumor Tissue (in manuscript).

effect in a tissue like the Flexner-Jobling carcinoma, in which the ratio of ATP-ase to Krebs cycle oxidation is much higher than in liver or kidney.

In attempting to rationalize the fact that the tumors have low levels of oxidative enzymes, as determined by a number of valid assays by the homogenate technic, but exhibit oxidative rates in slices comparable to those in liver and kidney, we have been led to the conclusion that the main rate-limiting factor in the tumor slice is the amount of the oxidative enzymes, while in the liver or kidney slice the oxidative enzymes are in excess and the rate-limiting factor is either substrate, phosphate acceptor, or inorganic phosphate (cf. 11, 21). This interpretation of the data obtained by the slice and by the homogenate technic has important implications regarding the uncontrolled growth of tumors, since if correct it would mean that building blocks for growth might be available at higher concentrations in tumor tissue than in nongrowing normal tissues, as indicated in Chart 3.

Since the studies with the homogenate and the slice appear to be measuring different properties, it was of interest to explore further the possibilities in the whole animal by use of a technic (15) based on earlier findings by Buffa and Peters (1). Potter and Busch (14) had shown that following the injection of fluoroacetate, spleen and thymus accumulated large amounts of citrate, while tumors and liver accumulated none. Yet spleen and thymus had no more oxidative enzymes than tumors, while liver had a great deal more of these enzymes than tumors, as proved by the homogenate technic. Further studies have now been carried out on liver, since it could be shown that great variations could be obtained experimentally. It has been found that low citrate values following the fluoroacetate injection are found only in males, not in females. However, if the females are fasted the value falls to that of the males, and then rises. On the other hand if the males are placed on a low protein diet their livers develop a transient ability to accumulate citrate.[3] These results suggest that the operation of the Krebs cycle in normal tissues is modified *in vivo* by hormone or other factors not yet apparent in slices or homogenates. Thus far tumors have not been found to accumulate citrate in the fluoroacetate test, but further studies on both hormones and other *in vivo* factors are in progress.

In summary, the following points may be taken to represent our interpretation of the data available up to this point:

1. Both slice and homogenate technics are in agreement that the enzymes of the Krebs cycle and the oxidative pathways are not missing from tumor tissues.

2. The homogenate technic shows that a limiting enzyme or group of enzymes in the oxidative complex is in tumors in amounts (*capacities*) roughly equivalent to the levels in spleen, thymus, lung, and embryonic tissue, and at levels *much lower* than are found in liver, kidney, and heart.

3. Since these differences are not apparent in the Q_{O_2} or the $Q_{C^{14}O_2}$ of tissue slices, the slice experiments do not measure the relative amounts (*capacities*) of these enzymes in the various tissues.

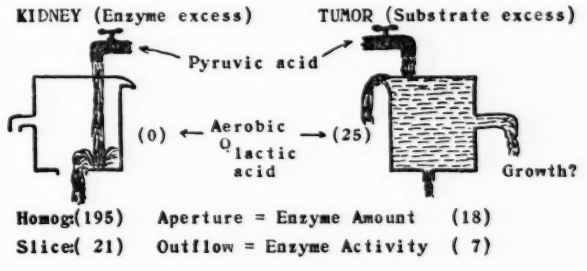


CHART 3.—Comparison of data from slices and homogenates. The figures 195 and 18 refer to the succinoxidase Q_{O_2} values for homogenates of rat kidney and Flexner-Jobling tumor, respectively, while the figures 21 and 7 refer to the Q_{O_2} of the two tissues using the slice technic with pyruvate as substrate.

4. Experiments with whole animals using fluoroacetate to block citrate oxidation *in situ* suggest that the enzyme activity of tissues *in situ* may be different than *either* the homogenate *or* slice would indicate at this time.

5. Attempts to obtain citrate accumulation in tumor tissue following fluoroacetate injection have been thus far unsuccessful, suggesting that one or more of the factors that limit citrate accumulation *in situ* is operative in tumors under conditions thus far observed.

6. Since both the homogenate and the slice technics indicate that the enzymes of the Krebs cycle are present and probably no lower in tumor than in spleen and thymus, the failure of the tumor to accumulate citrate following fluoroacetate remains unexplained. However, the experiments with liver in male and female rats demonstrate that, in the whole animal, hormone-enzyme interactions may modify enzyme activity in a way that overshadows the differences seen in slices or homogenates.

7. The comparison of results obtained by means of homogenates, slice technics involving isotopic

[3] V. R. Potter, H. Simonson, and R. K. Boutwell, Citrate Accumulation in Livers of Fasted and Protein-depleted Rats Following Injection of Fluoroacetate (in manuscript).

tracers, and whole animal studies lead inescapably to the conclusion that these studies measure different rate-limiting phenomena, and that any explanation of cancer metabolism based on one technic must be examined by the supplementary data provided by the other technics.

ACKNOWLEDGMENTS

Many of the experiments summarized in this report have depended on adequate supplies of adenosinetriphosphate and diphosphopyridine nucleotide, and we are indebted to Dr. G. A. LePage for supplying these compounds. We are also indebted to Drs. J. A. and E. C. Miller for supplying primary hepatomas for certain experiments, to Dr. LePage for supplying transplantable tumors, and to Dr. H. P. Rusch and all of the members of the McArdle Laboratory for helpful criticism and interest.

REFERENCES

1. Buffa, P. A., and Peters, R. A. Formation of Citrate *in Vivo* Induced by Fluoroacetate Poisoning. Nature, **163**:914, 1949.
2. Carruthers, C., and Suntzeff, V. Succinic Dehydrogenase and Cytochrome Oxidase in Epidermal Carcinogenesis Induced by Methylcholanthrene in Mice. Cancer Research, **7**:9–14, 1947.
3. Cori, C. F., and Cori, G. T. The Carbohydrate Metabolism of Tumors. II. Changes in the Sugar, Lactic Acid and CO_2-combining Power of Blood Passing through a Tumor. J. Biol. Chem., **65**:397–405, 1925.
4. ———. The Carbohydrate Metabolism of Tumors. III. The Rate of Glycolysis of Tumor Tissue in the Living Animal. J. Cancer Research, **12**:301–13, 1928.
5. DuBois, K. P., and Potter, V. R. Biocatalysts in Cancer Tissue. I. Cytochrome c. Cancer Research, **2**:290–93, 1942.
6. Elliott, K. A. C.; Benoy, M. P.; and Baker, Z. The Metabolism of Lactic and Pyruvic Acids in Normal and Tumor Tissues. II. Rat Kidney and Transplantable Tumours. Biochem. J., **29**:1937–50, 1937.
7. LePage, G. A. Phosphorylated Intermediates in Tumor Glycolysis. III. Effects of Anoxia and Hyperglycemia. Cancer Research, **8**:201–2, 1948.
8. Mider, G. B.; Tesluk, H.; and Morton, J. J. Effects of Walker Carcinoma 256 on Food Intake, Body Weight, and Nitrogen Metabolism of Growing Rats. Acta de l'Union internationale contre le cancer, **6**:409–20, 1948.
9. Olson, R. E., and Stare, F. J. The Metabolism of Radioactive Glucose and Pyruvate in Rat Hepatoma and Normal Liver. Abstracts, Am. Chem. Soc., 116th meeting, 61C, 1949.
10. Pardee, A. B.; Heidelberger, C.; and Potter, V. R. The Oxidation of Acetate-1-C^{14} by Rat Tissue *in Vitro*. J. Biol. Chem., **186**:625–35, 1950.
11. Potter, V. R. Phosphorylation Theories and Tumor Metabolism in Respiratory Enzymes, pp. 233–34. Madison: University of Wisconsin Press, 1942.
12. ———. The Assay of Animal Tissues for Respiratory Enzymes. IV. Cell Structure in Relation to Fatty Acid Oxidation. J. Biol. Chem., **163**:437–46, 1946.
13. ———. The Assay of Animal Tissues for Respiratory Enzymes. V. The Malic Dehydrogenase System. *Ibid.*, **165**: 311–24, 1946.
14. Potter, V. R., and Busch, H. Citric Acid Content of Normal and Tumor Tissues *in Vivo* Following Injection of Fluoroacetate. Cancer Research, **10**:353–56, 1950.
15. Potter, V. R.; Busch, H.; and Bothwell, J. Method for the Study of Tissue Metabolism *in Vivo* Using Fluoroacetate. Proc. Soc. Exper. Biol. & Med., **76**:38–41, 1951.
16. Potter, V. R., and Heidelberger, C. Alternative Metabolic Pathways. Physiol. Rev., **30**:487–512, 1950.
17. Potter, V. R., and Klug, H. L. Dietary Alteration of Enzyme Activity in Rat Liver. Arch. Biochem., **12**:241–48, 1947.
18. Potter, V. R., and LePage, G. A. Metabolism of Oxalacetate in Glycolyzing Tumor Homogenates. J. Biol. Chem., **127**:237–45, 1949.
19. Potter, V. R.; LePage, G. A.; and Klug, H. L. The Assay of Animal Tissues for Respiratory Enzymes. VII. Oxalacetic Acid Oxidation and the Coupled Phosphorylations in Isotonic Homogenates. J. Biol. Chem., **175**:619–34, 1948.
20. Potter, V. R., and Lyle, G. G. Oxidative Phosphorylation in Homogenates of Normal and Tumor Tissues. Cancer Research, **11**:355–60, 1951.
21. Potter, V. R.; Recknagel, R. O.; and Hurlbert, R. B. Intracellular Enzyme Distribution; Interpretations and Significance. Fed. Proc. (in press).
22. Potter, V. R., and Reif, A. E. Titration of a Succinoxidase Component by Antimycin. Fed. Proc., **10**:234, 1951.
23. Recknagel, R. O., and Potter, V. R. The Production of Ketone Bodies Induced by Ammonium Chloride. J. Biol. Chem. **191**:263–76, 1951.
24. Schneider, W. C., and Potter, V. R. Biocatalysts in Cancer Tissue. III. Succinic Dehydrogenase and Cytochrome Oxidase. Cancer Research, **3**:353–57, 1943.
25. Warburg, O. The Metabolism of Tumours. Translation by F. Dickens. London: Constable, 1930.
26. Weinhouse, S.; Millington, R. H.; and Wenner, C. E. Occurrence of the Citric Acid Cycle in Tumors. J. Am. Chem. Soc., **72**:4332–33, 1950.
27. Zamecnik, P. C. The Use of Labeled Amino Acids in the Study of Protein Metabolism of Normal and Malignant Tissues. Cancer Research, **10**:659–67, 1950.

Paper No. 2 in the Symposium on Intermediary Carbohydrate Metabolism in Tumor Tissue

Oxidation of C^{14}-labeled Carbohydrate Intermediates in Tumor and Normal Tissue*

ROBERT E. OLSON

(Department of Nutrition, Harvard School of Public Health and Department of Biological Chemistry, Harvard Medical School, Boston, Mass.)

Attempts to characterize the metabolic pattern in neoplastic tissue have occupied the attention of biochemists for over 4 decades. The search for a single distinguishing biochemical characteristic which is inclusive of all tumors and exclusive of all normal tissues has not been fruitful, although most tumors, regardless of histogenesis (17), tend to demonstrate a somewhat characteristic pattern in metabolizing glucose when studied *in vitro*. A high anaerobic glycolysis, a moderately high aerobic glycolysis, a moderately good Pasteur Effect, a moderately high respiration, and a respiratory quotient which is intermediate between 0.7 and 1.0 are some of the biochemical properties which are noted quite constantly in a high proportion of malignant tumors (6).

In his classical studies of tumor metabolism Warburg (53) placed great emphasis upon the relatively high rates of aerobic glycolysis shown by tumor slices as evidence for a defect in respiration which distinguished them from normal tissues. Dickens and Simer (10) then pointed out that tumors, in contradistinction to normal tissues (brain, retina, embryo) having a high anaerobic glycolysis, showed respiratory quotients of only 0.85 instead of 1.0 when incubated aerobically with glucose. Next it was shown that the concentration of a number of oxidative enzymes was reduced in various tumors. Schneider and Potter (47) reported that the succinic acid dehydrogenase, cytochrome c, and cytochrome oxidase activities of the rat hepatoma were only 22–30 per cent of those found in normal liver. Catalase was reported by Greenstein *et al.* (18) to be absent in transplanted hepatoma, and a number of flavin enzymes (D-amino acid oxidase, cytochrome c reductase, and xanthine dehydrogenase) have been found to be markedly reduced by other investigators (48, 40, 17). In addition to this, Pollack, Taylor, and Williams (37), Shack (48), and Kensler, Sugiura, and Rhoads (20) have shown that the level of certain vitamins of the B-complex and their associated coenzymes are greatly reduced in tumors. The data on this subject for the primary rat hepatoma are shown in Table 1.

TABLE 1

PERCENTILE B-VITAMIN AND COENZYME CONTENT OF HEPATOMA

VALUES FOR NORMAL LIVER = 100 PER CENT

Vitamin*	Per cent	Coenzyme	Per cent
Thiamine	28	Cocarboxylase	
Riboflavin	13	Flavin-adenine dinucleotide	17†
Nicotinic acid	17		
Pyridoxine	11	DPN	11‡
Pantothenic acid	10	Pyridoxal-PO_4§	
Biotin	10	Coenzyme A	33‖

References:
* (37)
† (48)
‡ (20)
§ Cohen *et al.* (8) have shown that transaminase in hepatoma is reduced to 30 per cent of that found in normal liver.
‖ (34)

It was this peculiarity of tumor tissue which first attracted our attention to the problem of tumor metabolism 2 years ago, at which time we were concerned experimentally with the effects of vitamin deficiency and depressed tissue coenzyme levels upon the metabolism of surviving tissues (28–30, 33). As seen in Table 1, six vitamins of the B-complex occur in hepatoma to the extent of only 10–28 per cent of their concentration in normal liver. This amount of depletion of a vitamin in a normal tissue almost invariably leads to changes

* Supported in part by grants-in-aid from the National Cancer Institute, The National Institutes of Health, Public Health Service, Bethesda, Md.; The American Cancer Society, via an Institutional Grant to Harvard University; and the Nutrition Foundation, Inc., New York, N.Y.

in tissue metabolism, and it seemed not improbable that if a tissue undergoing carcinogenesis lost up to 90 per cent of its vitamin and coenzyme content, some of the alterations in metabolism found in the neoplastic tissue might be explicable on this basis. On the other hand, it seemed unwarranted to draw conclusions about the efficacy of any given reaction in tumor tissue without exhaustive study, since the malignant tumor, despite its apparent deficiencies, appears to metabolize and grow in a highly effective manner.

Previous studies in our laboratory had shown that the effect of depletion of a given coenzyme in heart muscle by dietary restriction of the appropriate vitamin in rats and ducks gave unpredictable effects upon the metabolism of surviving tissue preparations. In thiamine (30) and biotin (29) deficiencies, the oxidation of pyruvate was markedly reduced in slices, whereas in pantothenic acid deficiency (33), even with reduction of cardiac coenzyme A to 20 per cent of normal, there was only a minimal effect upon pyruvate oxidation when studied with C^{14}-labeled pyruvate. When pyruvate oxidation was studied in homogenates of pantothenic acid-deficient heart muscle, however, a marked defect, particularly in the oxidation of the C_2-fragment derived from pyruvate, was observed. It was shown, furthermore, in homogenates of deficient ventricle, that citrate formation from pyruvate and fumarate was markedly reduced and could be restored by addition of coenzyme A (27). It appeared, therefore, that the enzymic architecture of the slice (and presumably the whole organ) was such that depletion of one factor required in the oxidative process might occur without serious effect, while the depletion of another co-factor required in a different step might seriously inhibit the same oxidative process. Comparative study of both slices and homogenates in deficient tissues where respiration was maintained in slices also seemed indicated. A study of hepatoma from this point of view seemed particularly attractive because of the moderately good respiration observed in slices of this tumor (6), despite the general poverty of respiratory catalysts and coenzymes.

In studying the metabolism of oxalacetate and pyruvate in fortified homogenates, Potter, LePage, and Klug (42) noted that while preparations of brain, liver, kidney, and heart tissue respired well in the presence of these acids, those of the primary hepatoma and two other tumors did not. Even with an actively glycolyzing system to maintain ATP levels in the tumor homogenate, the oxidation of oxalacetate was found to be negligible (41). Since the oxygen uptake reported for these experiments with tumor homogenates was much lower than that observed in slices of these tumors, it occurred to us that these discrepant results might be analogous to those obtained by us with homogenates and slices of pantothenic acid-deficient cardiac muscle slices. In this situation it was apparent that, although the depression of tissue coenzyme A levels was not sufficient to depress the oxidation of pyruvate in the slice, upon homogenization of the tissue with further dilution, the concentration of coenzyme A became limiting. Since the hepatoma is depleted not only of many coenzymes but also of certain apoenzymes, it seemed not unreasonable that its respiration should be abolished by homogenization.

Studies of the comparative respiration of homogenates and slices of the primary rat hepatoma were thus undertaken. It also seemed important to carry out a series of studies of the rate of oxidation of glucose and a number of carbohydrate intermediates labeled with radioactive carbon by slices of tumor, since, to our knowledge, no such study had been described in the literature. The use of tagged substrate, furthermore, seemed to provide the perfect opportunity to determine the actual magnitude of the oxidative pathway in tumor tissue and attempt to define the nature of the "defect," if any, which has been purported to exist in tumor respiration. The fate of labeled glucose, pyruvate, natural and unnatural lactate, acetate, and succinate have been studied in order to determine the disposition of substrate at various stages in the scheme of carbohydrate breakdown. Varying initial concentrations of substrate were used in order to evaluate mass action effects.

PROCEDURE

The tumor used in this study was the primary hepatoma induced by feeding young rats of the Sprague-Dawley (Hisaw) strain *m'*-methyl-*p*-dimethylaminoazobenzene at a level of 0.06 per cent in a diet moderately low in protein (12 per cent casein) and riboflavin (2 mg/kg) (15). The rats were kept on the carcinogenic diet for 3 months and then fed Purina Chow until hepatic tumors were palpable, at which time the animals were sacrificed for *in vitro* metabolic studies. Control animals received the experimental diet less azo dye for 3 months and then Purina Chow until used. Liver slices from fasted control rats were used as the control normal tissue in all experiments, and heart, brain, and liver slices from fed control rats were used in a few experiments. Only white, firm, grossly non-necrotic hepatoma nodules were selected for experiments with tumor tissue, and upon microscopic examination these pre-

sented the pathological picture of mixed hepatoma-cholangioma, with the ratio of hepatoma to cholangioma cells 7:1 or higher. Figure 1 shows a portion of a section of azo dye liver tumor which is almost exclusively hepatoma, and Figure 2 shows a portion of another section showing mainly the cholangioma formation.

The standard Warburg technic was employed in these experiments. Thin slices or homogenates of hepatoma and other normal tissues were incubated aerobically with radioactive substrates in a phosphate-saline medium (29) at 37° for 1 hour. The oxygen consumption was determined manometrically, the rate of oxidation of labeled substrate was determined by counting the activity of the $C^{14}O_2$ trapped in the center well with KOH, and the rate of substrate disappearance by applying either chemical (29), physical (36), or enzymatic technics (52) to the contents of the Warburg flask at the end of the period of incubation. Several radioactive substrates were used at concentrations ranging from 2.5 to 40 mM/l. Uniformly labled glucose-C^{14} was prepared photosynthetically from $C^{14}O_2$ as plant starch (23) and recrystallized with carrier after hydrolysis of the starch. Samples of glucose prepared by this method show only a very small percentile impurity when chromatographed on paper.[1] Uniformly labeled L(+)-lactate-C^{14} and D(−)-lactate-C^{14} were prepared by microbiological fermentation of evenly labeled glucose using *L. delbruckii* in the case of the natural isomer and *L. leishmanii* in the case of the unnatural isomer (3). These compounds were isolated as their zinc salts and characterized by rotation, water of crystallization, and carbon analysis. Acetate-1-C^{14} (carboxyl-labeled) and -2-C^{14} (methyl-labeled) were prepared from $C^{14}O_2$ and $C^{14}H_3I$, respectively (45). Pyruvate-2-C^{14} (carbonyl-labeled) and -3-C^{14} (methyl-labeled) were prepared from the acetate-1-C^{14} and -2-C^{14}, respectively, via acetyl halide and acetyl cyanide, with subsequent hydrolysis to yield pyruvic acid, which was then distilled, and the sodium salt recrystallized from dilute ethanol (33). Carboxyl-labeled succinate-C^{14} was prepared from unlabeled lithium acetylide by carbonation with $C^{14}O_2$ and subsequent catalytic reduction of the acetylenedicarboxylic acid (51). The final product melted at 190° and showed no depression when mixed with authentic succinic acid. The radioactivity of these compounds was determined by the wet combustion method of Van Slyke and Folch (57), and oxidation rates *in vitro* were determined by precipitating the $C^{14}O_2$ trapped in the center well as $BaC^{14}O_3$ which was counted on planchets with an end-window Geiger-Müller counter (29). All metabolic data including gas exchange, $C^{14}O_2$ production, and substrate utilization are expressed in terms of metabolic quotients (Q) which are defined as the number of μl. of gas or substrate (1 μM = 22.4 μl.) used or formed per milligram dry weight of tissue per hour. The final dry weight of the tissue was used as the basis for these calculations. The calculation of Net-$Q_{pyruvate}$ and of $Q_{C^{14}O_2}$ was carried out as previously described (29, 30).

[1] Private communication from Dr. Paul Zamecnik of the Massachusetts General Hospital.

TABLE 2

METABOLISM OF PYRUVATE IN SLICES AND HOMOGENATES OF HEPATOMA AND NORMAL LIVER*

Tissue	No. of experiments	Preparation	Q_{O_2} Pyruvate 5 mM/l	Values in terms of $Q_{pyruvate}$ Total Δ	Lactate formed	Net Δ	$C_{14}O_2$ (from 1-C^{14})	$C^{14}O_2$ (from 2-C^{14})
Hepatoma	12	Slice	7.0	−4.1	+1.8	−2.3	+1.8	+1.2
	4	Homogenate	1.5	−2.5	+2.3	−0.2	+0.1	trace
Liver	7	Slice	7.4	−5.0	+1.0	−4.0	+2.1	+0.9
	4	Homogenate	6.8	−4.0	+0.7	−3.3	+3.0	+0.4

* 0.5 Ml. of 10 per cent homogenate in KCl was added to prepared Warburg flasks containing 2.5 ml. phosphate-saline with pyruvate-1-C^{14} (5×10^{-3} M) or pyruvate-2-C^{14} (5×10^{-3} M), ATP (10^{-3} M) and cytochrome c (10^{-5} M). The gas phase for homogenate experiments was air; for slices, O_2.

Metabolism of pyruvate-1-C^{14} and -2-C^{14} in slices and homogenates of tumor and liver.—Preliminary studies of the comparative rates of oxidation of radioactive pyruvate in slices and homogenates of hepatoma and normal liver (34) showed that, while slices of hepatoma oxidized pyruvate to CO_2 at a good rate, homogenates of this tumor were inactive. Both slices and homogenates of liver tissue were able to oxidize pyruvate. These data are presented in Table 2. At a concentration of pyruvate of 5 mM/l, the liver slices showed remarkable ability to convert pyruvate to nonlactate products without loss of CO_2, the rate of disappearance of pyruvate to nonlactate products being almost twice the rate of decarboxylation of pyruvate. This capacity of liver and cardiac muscle slices (32) to carry out anabolic transformation of pyruvate is markedly reduced in hepatoma slices and is virtually absent in homogenates, as shown in Table 2. The tumor slice showed a faster rate of oxidation of C_2-fragments derived from added pyruvate (as

indicated by the greater recovery of $C^{14}O_2$ from pyruvate-2-C^{14}) than the liver slice, even though the total O_2 consumption was approximately the same. As previously noted by Potter, LePage, and Klug (42), homogenates of hepatoma fortified with ATP and cytochrome c did not respire appreciably or oxidatively catabolize pyruvate, the small amount of pyruvate disappearance being accounted for by reduction to lactate. Besides blocking anabolic reactions of pyruvate, homogenization of liver resulted in an increased rate of decarboxylation of pyruvate and and a decreased rate of oxidation of the C_2-fragment with about the same oxygen consumption as in the slice.

Metabolism of pyruvate-1-C^{14}, -2-C^{14}, and -3-C^{14} in slices of hepatoma and normal liver.—The terminal reactions in the oxidation of carbohydrate in hepatoma slices, as studied with 2-, 3- and 4-carbon acids shall be considered first. Certain data on the metabolism of pyruvate-1-C^{14} and -2-C^{14} in hepatoma slices shown in Table 2 have already been discussed in connection with the parallel studies on homogenates. In Chart 1 more complete information about the metabolism of all three radioisomers of pyruvate in hepatoma and liver at initial concentrations of substrate ranging from 2.5–40 mM/l is presented. Data on Q_{O_2} and $Q_{C^{14}O_2}$ are presented in the lower graphs, and data on disposition of substrate are shown in the upper graphs. The top curve in the upper graph represents total pyruvate disappearance as a function of initial concentration, the bottom curve represents the rate of conversion of pyruvate to lactate, and the intermediate cross-hatched area signifies the amount of pyruvate undergoing oxidation, as determined from the rate of decarboxylation of carboxyl-labeled pyruvate. The area bounded by the top curve and the next one below it, therefore, represent the conversion of pyruvate to nonlactate products without loss of CO_2. This fraction is regarded as the anabolic fraction and probably represents conversion of pyruvate to hexose, triose, or triose precursors via reactions of the glycolytic chain (33).

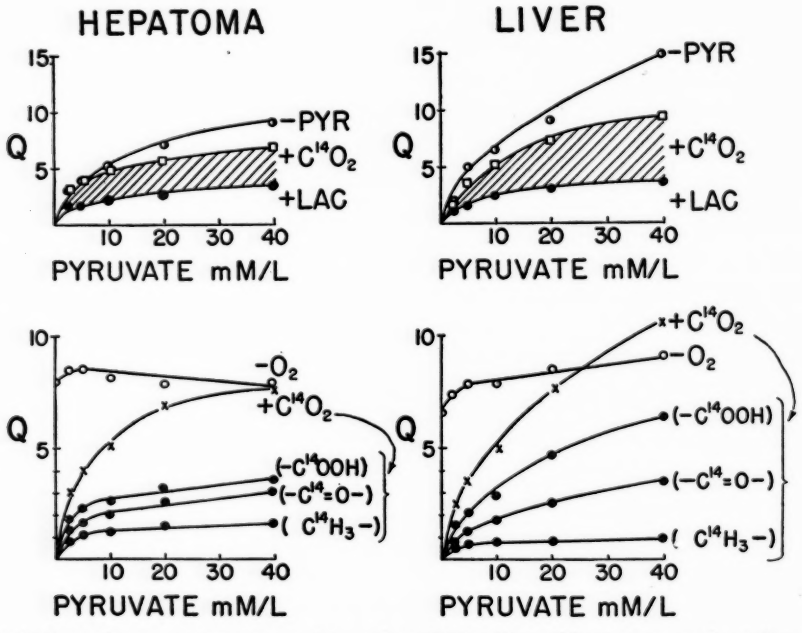


CHART 1.—The metabolism of pyruvate-1-C^{14}, -2-C^{14}, and -3-C^{14} in slices of hepatoma and rat liver. Metabolic quotient is plotted against substrate concentration. Disposition of substrate is presented in the top graphs and gas exchange in the bottom ones. The curve marked with (x) is a summation of the $C^{14}O_2$ produced from all three carbon atoms of labeled pyruvate. Each point represents at least four determinations.

Attempts to restore the oxidative capacity of homogenates of hepatoma by addition of liver boiled juice, of co-factors (in the following amounts per flask: cocarboxylase, 8 μg.; DPN, 50 μg.; TPN, 10 μg.; and coenzyme A, 20 acetylation units), or of fluoride (39) with or without co-factors were unsuccessful. It is possible that the absence of unknown co-factors, insufficient amounts of known co-factors, a deficiency in oxidative apoenzymes, or an excess of enzymes catabolic to ATP and co-factors are responsible for this failure. It is of great interest, however, that despite the impotence of even the fortified homogenate in oxidizing pyruvate to CO_2, the intact hepatoma cell is fully able to carry out this oxidation, which involves the co-ordination of many oxidative enzymes present in low amounts in this tumor.

There are both similarities and significant dif-

ferences in the response of hepatoma and liver to increasing concentrations of pyruvate. In both cases the total utilization of pyruvate and the production of $C^{14}O_2$ are positive functions of initial concentration of substrate. The total utilization of pyruvate in hepatoma was 50 per cent greater than in liver at 2.5 mM/l and 35 per cent less at 40 mM/l. The families of curves describing $C^{14}O_2$ production from each of the differently labeled isomers of pyruvate show interesting differences. In liver the three curves are widely separated, suggesting significant diversion of substrate carbon from the main oxidative pathway at several steps in the reaction sequence. Diversion of C_2-fragments from pyruvate into ketone body synthesis, lipogenesis, and other acylations (1) undoubtedly accounts for part of the difference in the recovery of $C^{14}O_2$ from pyruvate-2-C^{14} and -3-C^{14}. Another factor operating to reduce the recovery of $C^{14}O_2$ from pyruvate-2-C^{14} and -3-C^{14} is extensive exchange between the acids of the Krebs tricarboxylic acid cycle and nonisotopic compounds in equilibrium with them. In hepatoma slices the curves for recovery of $C^{14}O_2$ from the differently labeled pyruvates are much closer together, suggesting that diversion or exchange of the oxidative intermediates with nonisotopic endogenous compounds is considerably less in tumor tissue than in liver. It is also of interest that, although the rate of oxidation of the C_2-fragment derived from pyruvate is more rapid in tumor at 5 mM/l (see also Table 2), the rates of its oxidation in the two tissues at 40 mM/l are identical. The total $C^{14}O_2$ produced from added pyruvate was greater in liver than in tumor at high concentrations of substrate; at 5 mM/l the combustion of added substrate accounted for 50 per cent of the observed oxygen consumption in both tissues, and at 40 mM/l for about 88 per cent of the total oxygen consumption in tumor and 97 per cent of the observed oxygen consumption in liver. Respiratory quotients based upon total CO_2 production were not determined, but values based upon $Q_{C^{14}O_2}$ at 40 mM/l of pyruvate were 0.97 in tumor and 1.17 in liver slices. It is of interest that, although liver slices demonstrated anabolic transformations of pyruvate at all concentrations of substrate, such transformations were not evident in tumor slices until the pyruvate level was raised to 10 mM/l, and even at 40 mM/l reached only 40 per cent of that observed in liver. Another interesting effect of increasing pyruvate concentrations in hepatoma slices is the slight but definite lowering of the oxygen consumption which, in a tissue handicapped by lowered concentrations of hydrogen transport enzymes, might be the result of competition between pyruvate or anabolic products of pyruvate and oxygen for available hydrogen or electrons.

Many years ago Elliot, Benoy, and Baker (12) found that pyruvate at 20 mM/l resulted in a disappearance of pyruvate and stimulation of the R.Q. above unity in slices of Philadelphia No. 1 sarcoma and Walker carcinoma 256. Although they interpreted their data to indicate a limited conversion of pyruvate to succinate, these data, taken in conjunction with our own, strongly suggest that some tumors (perhaps all) have a sizeable aerobic pathway for the terminal oxidation of carbohydrate.

Metabolism of uniformly labeled L(+)- *and* D(−)*-lactate-*C^{14} *in slices of hepatoma and normal liver.*—Data bearing on the metabolism of natural lactic acid by tumor and liver are presented in Chart 2. As in the case of pyruvate, increasing concentrations of L(+)-lactate resulted in an increased utilization of this substrate in both tissues. Although utilization was equal in tumor and liver at 5 mM/l, tumor lagged by 30 per cent at 40 mM/l. Keto acid appearance, which apparently accounted for some of the lactate which disappeared, Q_{O_2}, and $C^{14}O_2$ production increased slightly with increases in concentration of substrate in both tumor and liver. The absolute rates of oxidation of L(+)-lactate in tumor and liver were almost identical at all levels of substrate and accounted for about 30 per cent of the observed oxygen consumption. The amount of lactate undergoing oxidation beyond pyruvate was calculated from the $Q_{C^{14}O_2}$ values in Chart 2 by referral to the curves for $Q_{C^{14}O_2\,(total)}$ and the $Q_{C^{14}O_2\,(carboxyl)}$ in Chart 1, and plotted on the upper diagram in Chart 2 as the cross-hatched area. It was of some interest that, although lactate was oxidized only $\frac{1}{3}$ as fast as pyruvate at comparable concentrations, it was anabolized 3–4 times as well as pyruvate in both liver and tumor. Although the depressing effect of substrate load upon Q_{O_2} in tumor was not noted with lactate as substrate, it is doubtful that the better anabolism of lactate is due only to the presence of two extra hydrogen atoms, since a more active anabolism of lactate was also noted in liver. The level of keto acid in these experiments in no way approaches the levels obtained when pyruvate was added as substrate, and yet the rate of anabolic transformation is greater with lactate. These data and certain others obtained by Miller (24) and Brin (3) suggest that a pathway of lactate toward phosphoglyceric acid which circumvents the pyruvate pool may be present in both tumors and normal tissues. Elliot, Benoy, and Baker (12) observed negligible effects of DL-lactate on R.Q. and Q_{O_2} of Philadelphia sarcoma and Walker 256

tumor, although small amounts of substrate disappeared.

From the historical point of view the data on oxidation of lactate-C^{14} are of interest in connection with the calculation of the Meyerhof Oxidation Quotient (5) as a measure of the Pasteur Effect. This formulation attempted to relate the decrease in glycolysis occurring when a tissue was changed from an anaerobic condition to an aerobic one to the rate of lactic acid oxidation under aerobic conditions, assuming that lactate oxidation accounted for the entire observed oxygen consumption, viz: $\text{M.O.Q.} = (Q^{N_2}_{CO_2} - Q^{O_2}_{CO_2}) / \frac{1}{3}Q_{O_2}$. Although the measurement of the Pasteur Effect in terms of resynthesis is now an outmoded procedure (7, 19, 22), it is of interest that on the basis of true lactate oxidation the M.O.Q. becomes 3–4 times as large. The M.O.Q. for the hepatoma is 2.9 calculated by the classical procedure (6) and 10.1 calculated on the basis of true lactate oxidation. This latter value is high enough to cast additional doubt (22) upon the hypothesis of resynthesis as an explanation of the Pasteur Effect.

TABLE 3

LIPOGENESIS FROM GLUCOSE-C^{14} AND ACETATE-C^{14} IN TISSUE SLICES

Tissue	Substrate	Radioactivity (cpm/mg/10^5 icpm)* FA	Cholesterol
Hepatoma	Glucose	224	28
	Acetate	128	43
Liver (fed)	Glucose	15	9
	Acetate	150	78

* ICPM = initial counts per minute of substrate concentration of glucose = 5 mM/l; concentration of acetate = 10 mM/l; time = 3 hrs.; medium = Krebs-Ringers.

The unnatural D(−)-lactate is utilized only about $\frac{1}{4}$ as well as the natural isomer in both tumor and liver, as shown in Chart 3. The oxidation rate of this compound was also $\frac{1}{4}$–$\frac{1}{5}$ as rapid as that of L(+)-lactate. Less keto acid appeared. There was no effect upon oxygen consumption in the tumor and only a slight effect in liver. These effects may possibly be due to the relatively feeble action of a racemase (3) in these tissues.

Metabolism of acetate-1-C^{14} and -2-C^{14} in slices of hepatoma and normal liver.—Studies of acetate metabolism are shown in Chart 4. The rate of acetate disappearance increased with concentration of substrate in both tumor and liver, the absolute rate of disappearance being twice as rapid in liver as in tumor. The oxidation of acetate was only slightly more rapid in liver than in hepatoma. The C_2-fragment from acetate is oxidized only $\frac{1}{2}$ as fast as that derived from pyruvate (see Chart 1) in liver, hepatoma, and cardiac muscle (33). Walker sarcoma 256, in contrast to the rat hepatoma, is virtually unable to oxidize acetate-1-C^{14} to $C^{14}O_2$ (36). Part of the nonoxidative or anabolic utilization of acetate is undoubtedly directed toward lipogenesis and other acylations (1). The data in Table 3 are taken from a study (26) in which acetate-1-C^{14} was incubated aerobically with hepatoma and liver slices for 3 hours and the cholesterol and fatty acids isolated from the slices and counted. The figures suggest that the incorporation of acetate carbon into the fatty acids and cholesterol of hepatoma, though somewhat less rapid than in liver from fed rats, is nonetheless a very active process.

Metabolism of carboxyl-labeled succinate-C^{14} in slices of hepatoma and liver.—Succinic acid was chosen for study because of its membership in the tricarboxylic acid cycle and because of the general interest in the succinoxidase system in tumor tissue. Studies of the succinoxidase system in tumor homogenates have shown low values, as previously indicated (47). Previous studies of succinic acid oxidation in slices of tumor tissue have shown some variability (12, 13, 46), although in general the data have shown only small effects of succinate upon Q_{O_2} with correspondingly small values for substrate utilization. The metabolic data obtained with carboxyl-labeled succinate-C^{14} in slices of hepatoma and liver in the present study are shown in Chart 5. A double ordinate representing substrate disposition on the one hand and gas exchange on the other is so numbered that a change of 2 μl. of substrate is equivalent to a change of 1 μl. of O_2. This numbering is based upon the theoretical situation of the pure succinoxidase preparation catalyzing the following reaction:

$$\text{Succinate} + \tfrac{1}{2}\,O_2 \longrightarrow \text{Fumarate} + H_2O.$$

If the two lines for oxygen consumption and substrate utilization were to coincide in Chart 5, the slice would be functioning as a succinoxidase preparation with complete suppression of endogenous respiration.

In hepatoma there was a stimulation of oxygen consumption as succinate concentration was raised, although the effect was negligible after 20 mM/l was reached. The degree of stimulation of oxygen consumption corresponds quite closely to that expected for a one-step oxidation of the succinate which disappeared. The appearance of $C^{14}O_2$ under these conditions suggests, however, that the oxidation of a portion of this fumarate had proceeded to oxalacetate or beyond with a small inhibition of endogenous respiration. Small amounts of lactate and keto acid also appeared when hepatoma slices were incubated with succinate.

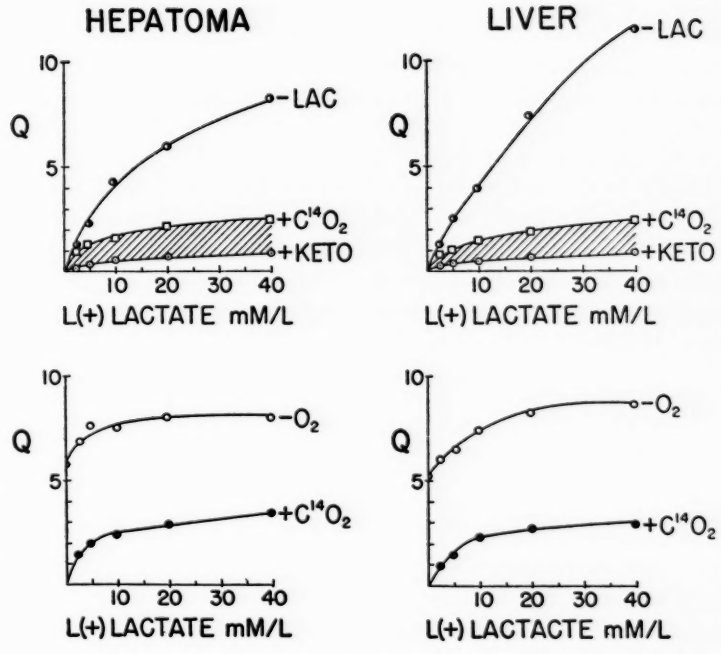


CHART 2.—The metabolism of uniformly labeled L(+)-lactate-C^{14} in slices of hepatoma and rat liver. Metabolic quotient is plotted against substrate concentration. Disposition of substrate is represented in the top graphs and gas exchange in the bottom ones. Each point represents at least four determinations.

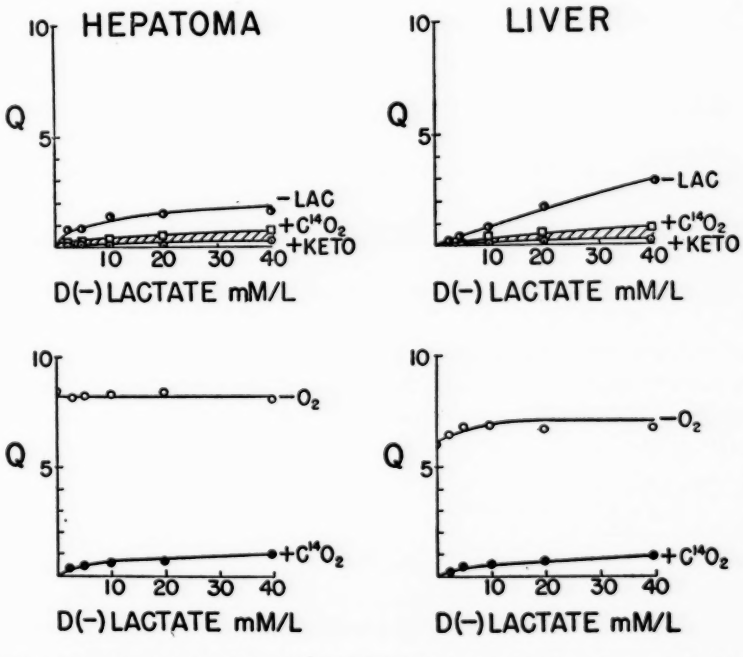


CHART 3.—The metabolism of uniformly labeled D (−) -lactate-C^{14} in slices of hepatoma and rat liver. Metabolic quotient is plotted against substrate concentration. Disposition of substrate is represented in the top graphs and gas exchange in the bottom ones. Each point represents at least four determinations.

As the succinate concentration was raised in experiments with liver slices, there was a steady rise in the Q_{O_2} from the endogenous value of 8 to 28, accompanied by a biphasic change in $C^{14}O_2$ production and in the accumulation of lactic and keto acids. In view of the close approximation of the lines for $-Q_{O_2}$ and $-Q_{succinate}$ at 40 mM/l, it seemed probable that at this concentration of succinate the liver slice was approaching the status of a succinoxidase preparation and pouring so much succinate hydrogen into the hydrogen transport system that other oxidations, including those of fumarate, were inhibited. Studies of the behavior of liver slices at higher concentrations of succinate-C^{14} are in progress in an attempt to delineate the relationship between succinic dehydrogenase and the cytochrome system in the intact liver cell.

By use of the data on succinic acid disappearance at 40 mM/l in Chart 5 as an index of succinoxidase activity, the ratio of activities obtained for hepatoma and liver slices is 10:52 (which is probably high, since no plateau was obtained in liver), compared to a ratio of 26:88 obtained in homogenates of these tissues by Schneider and Potter (47).

The metabolism of uniformly labeled glucose-C^{14} in slices of brain, heart, liver, and hepatoma.—An investigation of the oxidative metabolism of glucose-C^{14} in hepatoma slices seemed particularly desirable because of the extensive literature presenting evidence interpreted as consistent with the existence of a metabolic defect in the oxidation of glucose by tumor tissue (17). In a preliminary report we had noted, paradoxically, that the oxidation of radioactive glucose to $C^{14}O_2$ appeared to proceed faster in hepatoma than in liver from which it was derived. The data shown in Chart 6 confirm and extend these observations. In the upper graphs of Chart 6, the glucose uptake is plotted as triose in order to express the transformations of substrate in terms of C_3-fragments. The amount of C_3-compound undergoing decarboxylation was obtained from the $C^{14}O_2$ production in the same way as for lactate (*vide supra*) and plotted as the cross-hatched area. Aerobic glycolysis is represented by the area below the curve marked +Lac in the upper graphs.

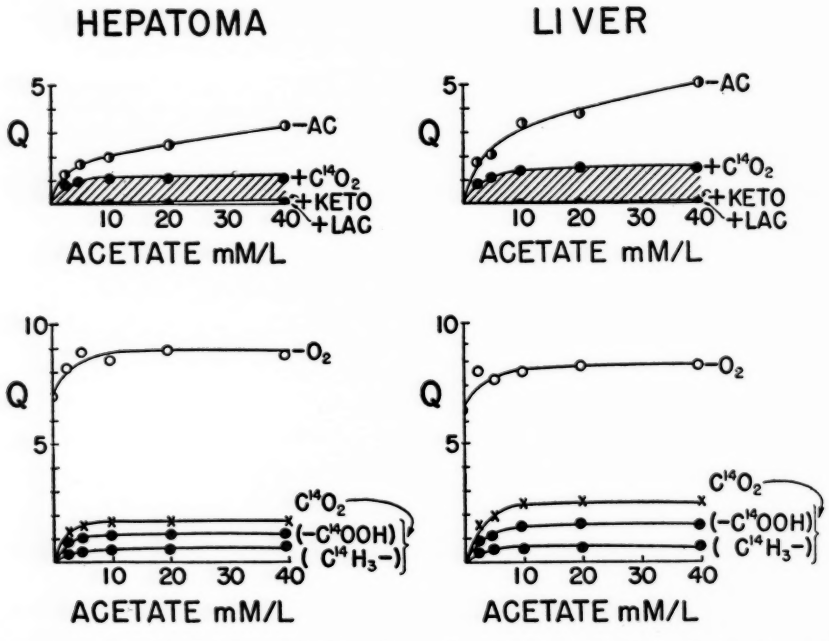


CHART 4.—The metabolism of acetate-1-C^{14} and -2-C^{14} in slices of hepatoma and rat liver. Metabolic quotient is plotted against substrate concentration. Disposition of substrate is presented in the top graphs and gas exchange in the bottom ones. The curve marked with (x) is a summation of $C^{14}O_2$ produced from both carbon atoms of labeled acetate. Each point represents at least four determinations.

As in the case of other substrates tested in this study, the rates of removal and oxidation of glucose increased with increasing concentrations of substrate, although in tumor and liver the rates were markedly different. In hepatoma slices about 10 per cent of the glucose which disappeared was oxidized to CO_2, and about 30 per cent appeared as lactate. In liver slices (from fasting rats), the uptake of glucose was lower and the oxidation and aerobic glycolysis almost negligible. In tumor, the combustion of glucose at a concentration of 10 mM/l accounted for about 30 per cent of the observed oxygen consumption, while in liver it accounted for about 2 per cent. At 10 mM/l, further-

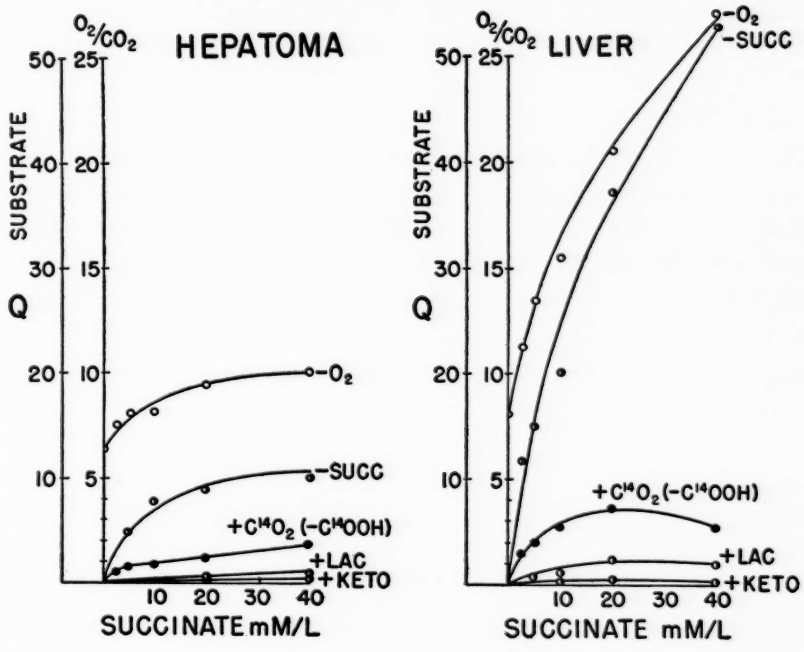


CHART 5.—The metabolism of carboxyl-labeled succinate-C^{14} in slices of hepatoma and rat liver. Metabolic quotient is plotted against substrate concentration. Substrate disposition and gas exchange are plotted on different scales on the ordinate so arranged that 1 μl. change in $O_2/C^{14}O_2$ exchange is equivalent to 2 μl. change in substrate utilization (see text). Each point represents at least four determinations.

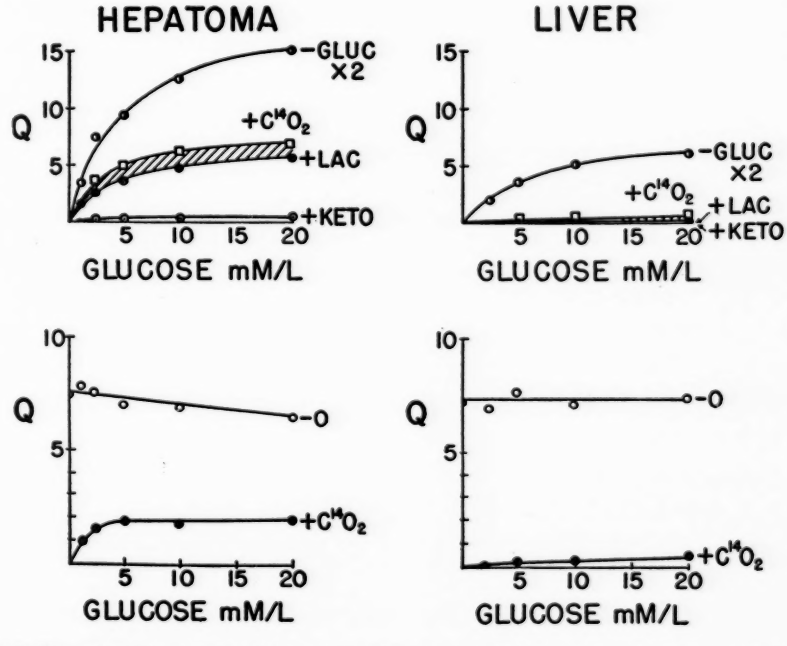


CHART 6.—The metabolism of uniformly labeled glucose-C^{14} in slices of hepatoma and rat liver. Metabolic quotient is plotted against substrate concentration. Disposition of substrate is presented in the top graphs and gas exchange in the bottom ones. Each point represents at least six determinations.

more, the rate of glucose combustion in tumor was 25 per cent of that of pyruvate and 75 per cent of that of L(+)-lactate at comparable substrate concentrations. The depression of Q_{O_2} with increasing concentrations of glucose in hepatoma is of some interest. It mirrors an effect noted by Elliot and Baker (11) in Philadelphia sarcoma and may be due to the diversion of ATP from oxidative processes to phosphorylation of glucose.

Since the oxidation of glucose in liver was singularly low, it appeared important to compare the hepatoma with other extra-hepatic tissues in regard to rates of glucose oxidation and aerobic glycolysis (31). These data are presented in Table 4. Brain slices which have an aerobic glycolysis

TABLE 4

AEROBIC GLYCOLYSIS AND OXIDATION OF GLUCOSE-C^{14} IN TISSUE SLICES

Species	Tissue	$+Q_{lactate}$	$+Q_{C^{14}O_2}$
Rat	Brain	4.5	10.5
	Heart	1.1	2.8
	Hepatoma	5.1	2.1
	Liver	0.0	0.2
Duck	Heart	1.2	2.5
	Liver	0.5	1.2

Phosphate-saline medium; glucose = 10 mM/l; gas phase = O_2.

comparable to hepatoma oxidized glucose to CO_2 5 times as fast as hepatoma, while heart slices which show a rate of glucose oxidation comparable to hepatoma accumulated lactate only $\frac{1}{5}$ as fast. Brain and heart thus showed a proportionality between the rates of glucose oxidation and aerobic glycolysis which hepatoma did not. It must be remembered, however, that the rate of lactate accumulation in tissue slices respiring under aerobic conditions merely reflects the degree of disparity between the activity of the glycolytic system and the activity of the system oxidizing pyruvate at the particular concentration of pyruvate present in the tissue. The data indicate that the activity of the terminal oxidation system (enzymes of the Krebs tricarboxylic acid cycle) is fully comparable to that of normal liver and cardiac muscle, though less active than that of brain. It is clear, however, that this system does not keep pace with the rate at which C_3-fragments are presented to it by the highly active glycolytic system of the hepatoma.

The finding of a very low rate of glucose oxidation in liver slices in the face of an active terminal oxidation system was very intriguing. The inertness of liver slices towards glucose as regards aerobic glycolysis, $C^{14}O_2$ production, and stimulation of respiration recalled the work of a number of investigators which had failed to show any effect of added glucose upon aerobic or anaerobic glycolysis in either slices (6, 35, 44, 54) or homogenates (21, 49) of liver. The finding of Stoesz and LePage (49) that hexose diphosphate was rapidly glycolyzed in homogenates of liver tissue, on the other hand, suggested that the enzymatic equipment for glycolysis in liver was intact from the level of aldolase on down. Likewise, the rapidity with which hexose forms glycogen in the intact rat (9), the ease with which radioactive glucose equilibrates with liver glycogen in liver slices (50), and the positive uptake of glucose in the present experiments with fasted liver slices, indicated that the enzymes concerned with the conversion of glucose to glycogen in liver tissue are active. It appeared, therefore, that the site of the physiological blockage of glycolysis in liver tissue was between glucose-6-phosphate and hexose diphosphate in the Embden scheme. Studies of the rates of anaerobic glycolysis in homogenates of many tissues, including hepatoma, with glucose-6-phosphate, fructose-6-phosphate, and hexose diphosphate as substrates indicated that, although fermentation of hexose-diphosphate was most active in all tissues, the most marked lag between the values obtained for hexose diphosphate and fructose-6-phosphate or glucose-6-phosphate was noted in rat liver (31). Assays for phosphohexokinase were then carried out on tissue extracts by the method of Racker (43) with the results shown in Table 5. Of the tissues tested, rat liver had the lowest content, with hepatoma about 3 times as active as liver. The fact that lipogenesis from glucose is more active in hepatoma than in rat liver, as shown in Table 3, is also consistent with the data on distribution of phosphohexokinase. It is of interest that duck liver, which is phylogenetically slightly more primitive, has a higher content of phosphohexokinase than rat liver, and that this is associated with increased ability of slices to oxidize and glycolyze added glucose (see Table 4).

TABLE 5

PHOSPHOHEXOKINASE ACTIVITIES OF TISSUE EXTRACTS

Species	Tissues	Units/ml extract*
Rat	Brain	100
	Heart	122
	Hepatoma	64
	Liver	22
Duck	Heart	140
	Liver	73

* Extract = supernatant of 10 per cent homogenate centrifuged at 3,000 rpm for 10 minutes.

Although Orr and Strickland (35) observed high rates of anaerobic glycolysis in livers rich in glyco-

gen from fed rats, a finding which has been questioned by Burk (6), we observed very little effect of high glycogen content upon aerobic glycolysis in our experiments, as shown in Table 6. The slightly greater endogenous lactate formation in liver slices from fed rats may represent breakdown of triose and other intermediates below the level of hexose diphosphate which may be present in greater quantity in livers from fed rats. Addition of C^{14}-glucose to slices of liver from fed rats did not materially affect the aerobic glycolysis, and the rate of its oxidation to $C^{14}O_2$ was somewhat less, as would be predicted from the dilution of added glucose-C^{14}, with glucose appearing in the medium through glycogenolysis of liver glycogen.

The synthesis of glycogen in liver slices from pyruvate, lactate, and CO_2 is well annotated (4). In our own studies the anabolism of pyruvate and lactate toward carbohydrate was more active in liver than in tumor. One may well inquire, assuming the activity of phosphohexokinase is limiting in glycolysis in liver, how the reverse reactions are successfully carried out. The answer is found in the fact that two enzymes are required for the interconversion of fructose-6-phosphate and fructose diphosphate, viz.:

$$\text{Fructose-6-PO}_4 \underset{\text{Phosphatase}}{\overset{\text{Phosphohexokinase + ATP}}{\rightleftharpoons}} \text{Fructose-1,6-(PO}_4)_2.$$

Inactivity of the phosphohexokinase system in no way vitiates the reverse reaction, because it is catalyzed by a different enzyme. This system is analogous, of course, to the hexokinase system, viz.:

$$\text{Glucose} \underset{\text{Phosphatase}}{\overset{\text{Hexokinase + ATP}}{\rightleftharpoons}} \text{Glucose-6-phosphate.}$$

The operation of these systems in normal liver is such that a conservation of glucose for secretion into the blood and nourishment of the extra-hepatic tissues is achieved. The relative inactivity of the phosphohexokinase system in liver serves as a ball valve to prevent hexose carbon, once synthesized from smaller molecules, or captured from the blood stream, from being broken down via the reactions of glycolysis. The oxidative requirements of the liver are apparently met by combustion of fat and 2- and 3-carbon acids.

General Conclusions about the Oxidative Pathway in Hepatoma Slices

Is there a "defect" in the respiration of tumor tissue as Warburg thought? In the hepatoma the magnitude of the pathway for oxidation of 2-, 3-, and 4-carbon intermediates of carbohydrate metabolism is within the range found for normal tissues, the values for hepatoma being very close to those found in liver. The relative rates of $C^{14}O_2$ production from acetate and pyruvate labeled in each of their carbon atoms in hepatoma slices, the isolation of radioactive citrate from tumors oxidizing radioactive fatty acids by Weinhouse, Millington, and Wenner (55), and the identification of most of the enzymes which catalyze reactions of the Krebs tricarboxylic acid cycle in tumor tissue (40, 47, 55) suggest, furthermore, that the mechanism of the terminal oxidation of substrate in tumor tissue is the same as in normal tissues. A defect in the efficiency of coupling of oxidation to high energy phosphate bond generation in tumor tissue cannot, however, be ruled out without further studies.

Biochemical Differences Between Liver and Hepatoma

A summary of some of the metabolic differences between liver and hepatoma is presented in Chart 7. Although from our studies there appears to be no absolute change in the qualitative aspects of metabolism in the hepatoma, in some cases, the degree of change, quantitatively, in the routing of metabolic traffic is almost tantamount to a qualitative change. In normal liver, glycogen is synthesized from blood glucose and blood lactate, stored, and resecreted as glucose. Very little is glycolyzed. As the liver undergoes neoplastic change, glycolysis increases, anaerobic before aerobic (25), until in the full-blown state the liver tumor is diverting all its glucose uptake into the glycolytic pathway, with a negligible storage of glycogen (17). Carbohydrate synthesis from pyruvate is also reduced. To account for this, our data suggest that both the hexokinase and phosphohexokinase systems become more active in the liver tumor and succeed in diverting glucose into glycolytic channels where glucose carbon becomes available for lipogenesis, amino acid synthesis, purine and pyrimidine synthesis, or further oxidation to CO_2. The rate of glycolysis exceeds the rate of utilization of C_3-fragments sufficiently to maintain a high concentration of C_3-reactants in the tumor tissue (38). The lower contents of oxidative

TABLE 6

Effect of Fasting and Feeding upon Glucose Metabolism of Rat Liver Slices*

State	Glycogen (per cent)	$Q_{lactate}$ (Endogenous)	$Q_{lactate}$ (Glucose)	$Q_{glucose}$ (Net)	$Q_{C^{14}O_2}$
Fasted	0.15	+0.35	+0.40	−2.52	+0.20
Fed	4.55	+1.35	+1.38	+8.54	+0.15

* Concentration of radioactive glucose in the medium = 10 mM/l. Each value is the mean of 6 determinations.

enzymes and coenzymes in the hepatoma apparently does not compromise the ability of this tissue to carry out the terminal oxidation of carbohydrate at rates comparable to that of the liver cell. Fewer side reactions appear to occur in the combustion of C_3-fragments and C_2-fragments via the tricarboxylic acid cycle in the hepatoma than in the liver. Despite oxidative activity, glycolysis continues, presumably because the breakdown of ATP for growth and other anabolic reactions cannot be matched by oxidative phosphorylation (38). Urea synthesis is abolished (17), and the tumor becomes vascularized with an arterial blood supply (2). In all respects the hepatoma has become an extra-hepatic tissue.

The work reported today constitutes only a small beginning in the application of tracer technics to the problem of the intermediary metabolism of tumor tissue. It seems to me that the working hypothesis for future work in this field should be that the biochemical differentiation of the tumor is peculiar to its needs and that the motivation for the clarification of that biochemical pattern be to understand its operation rather than merely to draw comparisons with a variety of normal tissues.

ACKNOWLEDGMENTS

The author is pleased to thank Heidi Richards, Mary Jones, and Mary Meadows for technical assistance in carrying out the experiments reported in this paper. He is indebted to Mr. John DiGiorgio for assistance in preparing some of the radioactive compounds used in this study and to Dr. Myron Brin for preparation of the radioactive antipodes of lactic acid. He is pleased to acknowledge the aid of Dr. James Goddard in examining and describing pathological sections of tumor tissue. This work was done during the tenure of a Research Fellowship of the American Heart Association.

REFERENCES

1. Bloch, K. The Metabolism of Acetic Acid in Animal Tissues. Physiol. Rev., **27**:574–620, 1947.
2. Breedis, C., and Young, G. Blood Supply of Neoplasms in the Liver. Fed. Proc., **8**:351, 1949.
3. Brin, M. A Study of the *In Vitro* Metabolism of Cardiac Muscle with Especial Reference to Pyridoxine Deficiency. Thesis, pp. 29–38. Harvard Univ., 1951.
4. Buchanan, J. M., and Hastings, A. B. The Use of Iso-

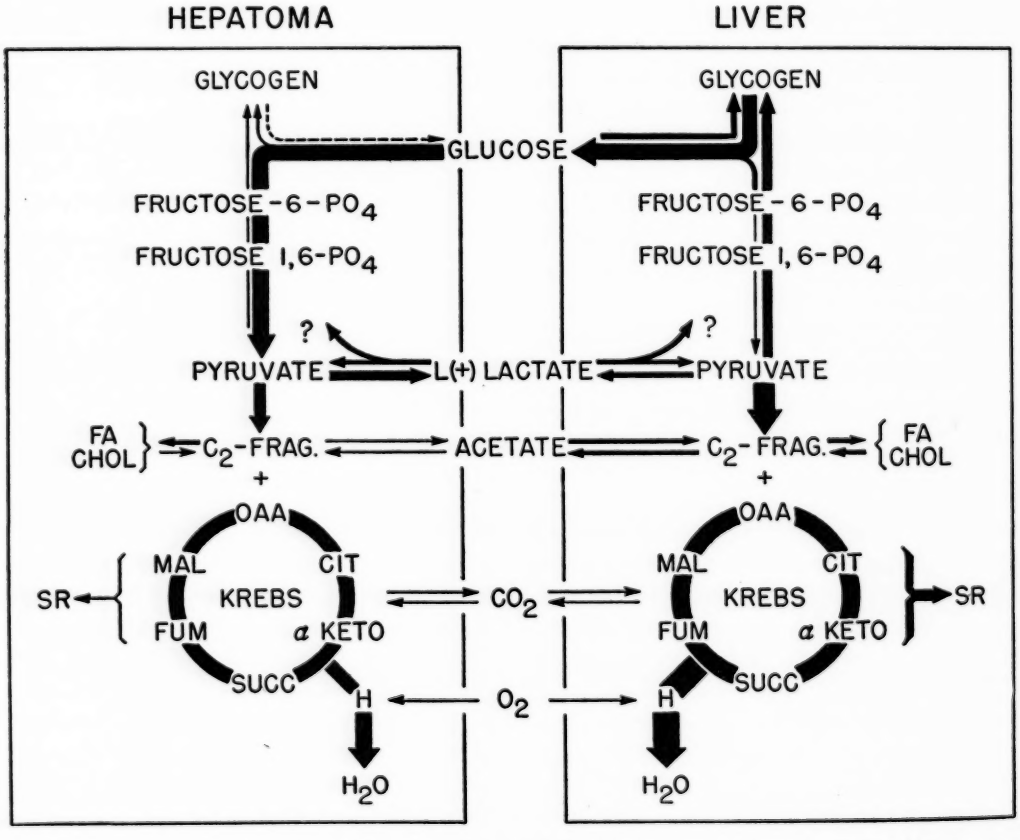


Chart 7.—Summary of the differences in carbohydrate metabolism between liver and hepatoma. The boxes represent adjacent tumor and liver cells and the space between them a blood vessel. The thickness of the lines represents the approximate reaction rates and the arrows their direction. The acids of the Krebs tricarboxylic acid cycle are abbreviated as follows: OAA = oxalacetate, CIT = citrate, α-KETO = α-ketoglutarate, SUCC = succinate, FUM = fumarate, and MAL = malate. Other abbreviations are: FA = fatty acids, CHOL = cholesterol, SR = side reactions.

topically Marked Carbon in the Study of Intermediary Metabolism. Physiol. Rev., **26**:120–55, 1946.

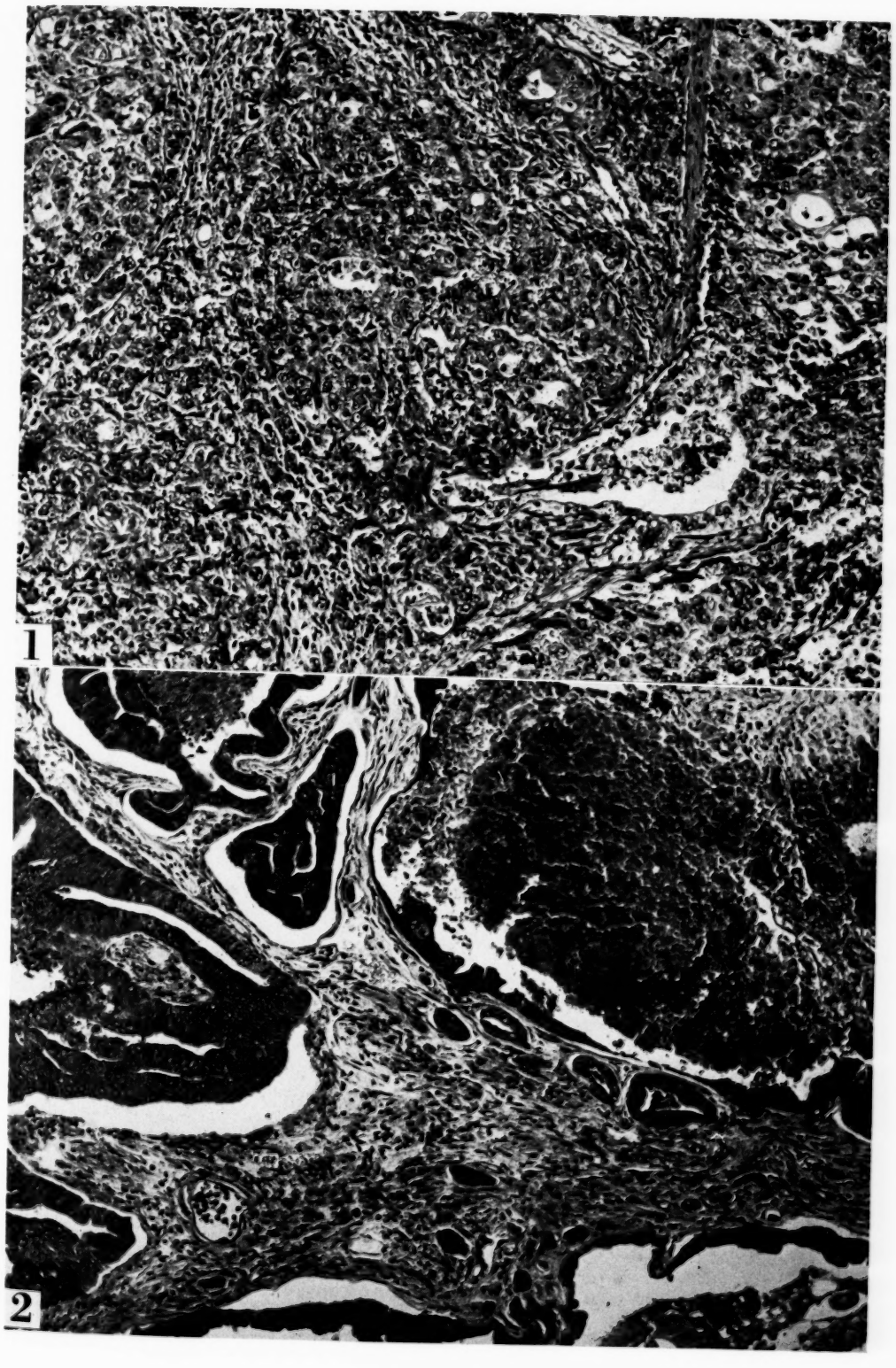
5. BURK, D. A Colloquial Consideration of the Pasteur and Neo-Pasteur Effect. Cold Spring Harbor Symp. Quant. Biol., **7**:420–59, 1939.
6. ———. On the Specificity of Glycolysis in Malignant Liver Tumors as Compared with Homologous Adult or Growing Tissues. Symposium on Respiratory Enzymes, pp. 235–45. Madison: University of Wisconsin Press, 1942.
7. BURK, D.; WINZLER, R. J.; and DU VIGNEAUD, V. The Role of Biotin in Fermentation and the Pasteur Effect. J. Biol. Chem., **140**:Proc., xxi–xxii, 1941.
8. COHEN, P. P.; HEKHUIS, G. L.; and SOVER, E. K. Transamination in Liver from Rats Fed Butter Yellow. Cancer Research, **2**:405–10, 1942.
9. CORI, C. F. The Rate of Glycogen Formation in the Liver of Normal and Insulinized Rats during the Absorption of Glucose, Fructose, and Galactose. J. Biol. Chem., **70**:577–85, 1926.
10. DICKENS, F., and SIMER, F. CXLIII. The Metabolism of Normal and Tumour Tissue. II. The Respiratory Quotient and the Relationship of Respiration to Glycolysis. Biochem. J., **24**:1301–26, 1930.
11. ELLIOT, K. A. C., and BAKER, Z. CCLXXXIX. The Respiratory Quotients of Normal and Tumour Tissue. Biochem. J., **29**:2433–41, 1935.
12. ELLIOT, K. A. C.; BENOY, M. P.; and BAKER, Z. CCXXIX. The Metabolism of Lactic and Pyruvic Acids in Normal and Tumour Tissue. II. Rat Kidney and Transplantable Tumours. Biochem. J., **29**:1937–50, 1935.
13. ELLIOT, K. A. C., and GREIG, M. E. The Distribution of the Succinic Oxidase System in Animal Tissues. Biochem. J., **32**:1407–23, 1938.
14. ———. CXXXIII. The Metabolism of Lactic and Pyruvic Acids in Normal and Tumour Tissue. IV. Formation of Succinate. *Ibid.*, **31**:1021–32, 1937.
15. GIESE, J. E.; MILLER, J. A.; and BAUMANN, C. A. The Carcinogenicity of *m'*-Methyl-*p*-Dimethylaminoazobenzene and of *p*-Monomethylaminobenzene. Cancer Research, **5**:337–40, 1945.
16. GOULD, R. G.; HASTINGS, A. B.; ANFINSEN, C. B.; ROSENBERG, I. N.; SOLOMON, A. K.; and TOPPER, Y. J. The Metabolism of Isotopic Pyruvate and Acetate in Rabbit Liver Slices *In Vitro*. J. Biol. Chem., **177**:727–31, 1949.
17. GREENSTEIN, J. P. Properties of Tumors. Biochemistry of Cancer, pp. 175–315, New York: Academic Press, 1947.
18. GREENSTEIN, J. P., EDWARDS, J. E.; ANDERVONT, H. B.; and WHITE, J. Comparative Enzymatic Activity of Transplanted Hepatomas and of Normal, Regenerating, and Fetal Liver. J. Nat. Cancer Inst., **3**:7–17, 1942.
19. JOHNSON, M. J. The Role of Aerobic Phosphorylation in the Pasteur Effect. Science, **94**:200, 1941.
20. KENSLER, C. J.; SUGIURA, K.; and RHOADS, C. P. Coenzyme I and Riboflavin Content of Livers of Rats Fed Butter Yellow. Science, **91**:623, 1940.
21. LEPAGE, G. A. A Comparison of Tumor and Normal Tissues with Respect to Factors Affecting the Rate of Anaerobic Glycolysis. Cancer Research, **10**:77–88, 1950.
22. LIPMAN, F. Pasteur Effect. Symposium on Respiratory Enzymes, pp. 48–71. Madison: University of Wisconsin Press, 1942.
23. LIVINGSTON, L. G., and MEDES, G. The Biosynthesis of C^{13}-Labeled Starch. J. Gen. Physiol., **31**:75–88, 1947.
24. MILLER, O. N. A Study of *In Vitro* Metabolism of Cardiac Muscle with Especial Reference to Niacin and Folic Acid Deficiency. Thesis, pp. 77–105, Harvard Univ., 1950.
25. NAKATANI, M.; NAKANO, K.; and OHARA, Y. Untersuchung über den Gewebestüffwechsel beim Verlauf der Leberkrebsentstehung durch Dimethyleminoazobenzolfütterung. Gann, **32**:240–44, 1938.
26. OLSON, R. E.; HIRSCH, E. G.; and JONES, M. C. Formation of Fatty Acids and Cholesterol from Radioactive Acetate and Glucose in Rat Heart, Liver, and Hepatoma. Fed. Proc., **10**:230, 1951.
27. OLSON, R. E.; HIRSCH, E. G.; RICHARDS, H.; and STARE, F. J. Coenzyme A and Citrate Formation in Homogenates of Heart Ventricle from Normal and Pantothenic Acid-Deficient Ducklings. Arch. Biochem., **22**:480–82, 1949.
28. OLSON, R. E., and KAPLAN, N. O. The Effect of Pantothenic Acid Deficiency upon the Coenzyme A Content and Pyruvate Utilization of Rat and Duck Tissues. J. Biol. Chem., **175**:515–29, 1948.
29. OLSON, R. E.; MILLER, O. N.; TOPPER, Y. J.; and STARE, F. J. The Effect of Vitamin Deficiencies upon the Metabolism of Cardiac Muscle *in Vitro*. II. The Effect of Biotin Deficiency in Ducks with Observation on the Metabolism of Radioactive-Carbon-Labeled Succinate. J. Biol. Chem., **175**:503–14, 1948.
30. OLSON, R. E.; PEARSON, O. H.; MILLER, O. N.; and STARE, F. J. The Effect of Vitamin Deficiencies upon the Metabolism of Cardiac Muscle *in Vitro*. I. The Effect of Thiamine Deficiency in Rats and Ducks. J. Biol. Chem., **175**:489–501, 1948.
31. OLSON, R. E.; ROBSON, J. S.; RICHARDS, H.; and HIRSCH, E. G. Comparative Metabolism of Radioactive Glucose in Heart, Brain, Kidney, and Liver Slices. Fed. Proc., **9**:211, 1950.
32. OLSON, R. E., and STARE, F. J. Metabolism of Radioactive Acetate and Pyruvate by Cardiac Muscle from Normal and Pantothenic Acid-Deficient Ducklings. Fed. Proc., **8**:391–92, 1949.
33. ———. Metabolism *in Vitro* of Cardiac Muscle in Pantothenic Acid Deficiency. J. Biol. Chem., **190**:149–64, 1951.
34. ———. The Metabolism of Radioactive Glucose and Pyruvate in Rat Hepatoma and Normal Liver. Absts. Am. Chem. Soc., 116th Meeting, 61c–62c, 1949.
35. ORR, J. W., and STRICKLAND, L. H. The Metabolism of Rat Liver during Carcinogenesis by Butter Yellow. Biochem. J., **35**:479–87, 1941.
36. PARDEE, A. B.; HEIDELBERGER, C.; and POTTER, V. R. The Oxidation of Acetate-1-C^{14} by Rat Tissue *in Vitro*. J. Biol. Chem., **186**:625–35, 1950.
37. POLLACK, M. A.; TAYLOR, A.; and WILLIAMS, R. J. B Vitamins in Human, Rat, and Mouse Neoplasms. U. of Tex. Pub., **4237**:56–71, 1942.
38. POTTER, V. R. Phosphorylation Theories and Tumor Metabolism. Symposium on Respiratory Enzymes, pp. 233–34. Madison: University of Wisconsin Press, 1942.
39. ———. Studies on the Reactions of the Krebs Citric Acid Cycle in Tumor with Homogenates, Slices, and *in Vivo* Technics. Cancer Research, **11**:565–70, 1951.
40. ———. The Assay of Animal Tissues for Respiratory Enzymes. V. The Malic Dehydrogenase System. J. Biol. Chem., **165**:311–24, 1946.
41. POTTER, V. R., and LEPAGE, G. A. Metabolism of Oxalacetate in Glycolyzing Tumor Homogenates. J. Biol. Chem., **177**:237–45, 1949.
42. POTTER, V. R.; LEPAGE, G. A.; and KLUG, H. L. The Assay of Animal Tissues for Respiratory Enzymes. VII. Oxalacetic Acid Oxidation and the Coupled Phosphorylations in Isotonic Homogenates. J. Biol. Chem., **175**:619–34, 1948.
43. RACKER, E. Spectrophotometric Measurement of Hexokinase and Phosphohexokinase Activity. J. Biol. Chem., **167**:843–53, 1947.
44. ROSENTHAL, O., Untersuchungen über Milchsäuregärung

von Warmblütergeweben. Biochem. Ztschr., **207**:263–97, 1929.

45. Sakami, W.; Evans, W. E.; and Gurin, S. The Synthesis of Organic Compounds Labeled with Isotopic Carbon. J. Am. Chem. Soc., **69**:1110–12, 1947.
46. Salter, W. T.; Craig, F. N.; and Bassett, A. M. Observations on the Oxidative Behavior of Tumors, Artificially Benign Tumors, and the Homologous Normal Tissues. Cancer Research, **1**:751, 1941.
47. Schneider, W. C., and Potter, V. R. Biocatalysts in Cancer Tissue. Cancer Research, **3**:353–57, 1943.
48. Shack, J. Cytochrome Oxidase and D-Amino Acid Oxidase in Tumor Tissue. J. Nat. Cancer Inst., **3**:389–96, 1943.
49. Stoesz, P. A., and LePage, G. A. Glycolysis in Liver Homogenates. J. Biol. Chem., **180**:587–95, 1949.
50. Teng, C.-T.; Sinex, F. M.; and Hastings, A. B. Factors Affecting Glycogen Formation *in Vitro*. Fed. Proc., **9**:237, 1950.
51. Topper, Y. J. Note on the Synthesis of Succinic Acid Labeled in the Carboxyl Position with Radioactive Carbon. J. Biol. Chem., **177**:303–4, 1949.
52. Umbreit, W. W.; Burris, R. H.; and Stauffer, J. F. Manometric Techniques and Related Methods for the Study of Tissue Metabolism, pp. 142–44, Minneapolis: Burgess Publishing Co., 1945.
53. Warburg, O. Über den Stoffwechsel der Tumoren. Berlin: Springer, 1926.
54. Warren, C. O., and Ebaugh, F. G., Jr. Effect of Various Ions on Anaerobic Glycolysis of Rat Liver *in Vitro*. Am. J. Physiol., **147**:509–16, 1946.
55. Wenner, C. E.; Spirtes, M. A.; and Weinhouse, S. Enzymes of the Citric Acid Cycle in Tumors. J. Am. Chem. Soc., **72**:4333, 1950.
56. Weinhouse, S.; Millington, R. H.; and Wenner, C. E. Occurrence of Citric Acid Cycle in Tumors. J. Am. Chem. Soc., **72**:4332–33, 1950.
57. Van Slyke, D. D., and Folch, J. J. Manometric Carbon Determination. J. Biol. Chem., **136**:509–41, 1940.

Fig. 1.—Rat liver tumor induced by feeding *m'*-methyl-*p*-dimethylaminoazobenzene. Zenkers fixative and Masson's trichrome stain with a green filter. Mag. ×770. This is almost exclusively hepatoma with very little necrosis. There is exuberant young fibrous tissue proliferation. A portal vein filled with tumor cells is seen to the right of center.

Fig. 2.—Rat liver tumor induced by feeding *m'*-methyl-*p*-dimethylaminoazobenzene. Zenkers fixative and Masson's trichrome stain with a green filter. Mag. ×770. This shows mainly cholangioma, the bile duct epithelial cells being involved in the neoplastic change. There is a marked stromal reaction and an area of necrosis at the upper right.


1
2

Paper No. 3 in the Symposium on Intermediary Carbohydrate Metabolism in Tumor Tissue

Studies on the Fate of Isotopically Labeled Metabolites in the Oxidative Metabolism of Tumors*

SIDNEY WEINHOUSE

(The Lankenau Hospital Research Institute and the Institute for Cancer Research, Philadelphia, Pa.)

Although biochemical investigation of the cancer problem has disclosed many quantitative differences between normal and neoplastic tissues, no characteristic exclusive chemical or metabolic property of tumor cells has emerged from such studies (6, 12). Because of the unregulated growth, which is an inherent feature of malignancy, attention has been focused particularly on the problem of energy production in tumors; and it is in this area of cancer research that metabolic exploration has perhaps penetrated most deeply. Modern research in the field of cellular metabolism of tumors began with the studies of Warburg (13) 30 years ago, with the advent of the tissue slice technic. These studies disclosed certain quantitative differences between normal and tumor tissues which as yet remain unexplained, but which represent perhaps the closest approach to what may be considered a distinctive metabolic pattern of tumor cells.

When normal tissues are sliced thinly and placed in a medium of suitable ionic composition, they will survive and continue various metabolic activities; they will, for example, absorb oxygen and liberate carbon dioxide uninterruptedly for many hours. In the presence of glucose and in the absence of oxygen they will produce variable amounts of lactic acid, but with oxygen present much less lactic acid is produced—a result which is not unexpected, since some lactic acid presumably would be removed by oxidation. Oddly, however, the decrease in lactic acid formation brought about by oxygen is much greater than can be accounted for simply by oxidation of lactic acid; in most instances it is of the order of 3–6 times this quantity. This phenomenon is known as the Pasteur Effect, being similar to the inhibition of fermentation by oxygen, observed many years ago by Pasteur in various microörganisms. Warburg (13) found that tumor tissues also displayed this Pasteur Effect and to about the same extent, quantitatively, as normal tissues. He also found that the rates of oxygen consumption, though on the low side, were within the range of those of normal tissues. In contrast to normal tissues, however, the level of lactic acid production was extremely high in tumors, so that even with a "normal" Pasteur Effect lactic acid production in the presence of oxygen was also high—much higher, even, than in most normal tissues in the absence of oxygen. A comparison of various normal and neoplastic tissues is given in Table 1.

TABLE 1

GLYCOLYSIS IN NORMAL AND NEOPLASTIC TISSUES
(Data of Warburg)

Tissue	Q_{O_2}*	$Q_L^{O_2}$	$Q_L^{N_2}$
Normal:			
Kidney, rat	21	0	3
Liver, rat	12	0.6	3
Pancreas, dog	3	0	4
Brain cortex, rat	11	2.5	19
Neoplastic:			
Jensen sarcoma, rat	9	17	34
Flexner-Jobling carcinoma, rat	7	25	31
Sarcoma 37, mouse	15	12	28

* Q values represent μl. of gas/mg dry tissue/hour.

At one time Warburg believed that in the high aerobic glycolysis he had uncovered the secret of malignancy. It developed subsequently, however, that this property is not exclusive with neoplasms but is displayed to varying extents by other growing tissues, so that the force of this finding has been blunted; nevertheless, it still remains one of

* This work was aided by grants from the National Cancer Institute, Public Health Service; the American Cancer Society, on recommendation by the Committee on Growth of the National Research Council; and the Atomic Energy Commission (Contract No. AT [30-1] 777).

the few well authenticated metabolic differences between normal and neoplastic cells.

The high aerobic glycolysis of tumors was attributed by Warburg to a disturbance in respiration, which somehow prevents the oxygen from exercising its control over glycolysis. This conception was supported and elaborated, principally by Dickens and his school (4), and has received wide attention up to the present time. In a masterly review of the Pasteur Effect and tumor metabolism, Burk (3) has shown that this viewpoint is probably a mistaken one; however, no alternate explanation for the high aerobic glycolysis of tumors has gained

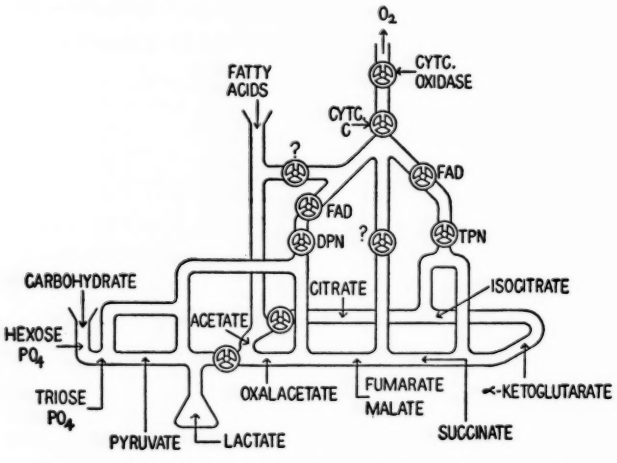


CHART 1.—Cellular engine for oxidation of carbohydrates and fatty acids. Horizontal pipes represent pathway of carbon transport; vertical pipes represent electron transport. Valves represent enzymatic steps, at which points "blocks" may occur.

general acceptance, and the view still prevails that neoplastic tissues are characterized by a deficiency or inadequacy of oxidative metabolism. Without taking any further time to discuss this hypothesis we might consider, in the light of our present knowledge of the respiratory metabolism of normal tissues, what kind of disturbances in respiration might lead to lactic acid formation. To do this I've constructed a cellular engine out of some pipes and valves, shown in Chart 1. The function of this engine is to provide energy by abstracting electrons from metabolites such as glucose or fatty acids and combining them with oxygen. The carbon atoms travel along the horizontal pipes. Glucose is phosphorylated and broken down to trioses, which are oxidized to pyruvic acid; and this in turn is oxidized to acetic acid, which, by condensation with oxalacetic acid, enters the citric acid cycle to be oxidized to CO_2 with the regeneration of oxalacetic acid.

The oxidation of glucose yields electrons, twelve for each triose molecule, and these come off in pairs of two at six places along the path: triose phosphate, pyruvate, isocitrate, α-ketoglutarate, succinate, and malate. Before they reach oxygen, these electrons must pass through the vertical pipes which represent the electron transport system, the known components of which are the pyridine nucleotides, flavin adenine nucleotide, and the cytochromes. We can assume that in the normal, functioning cell, all the valves are at least partially open, so that carbon atoms can travel unimpeded through the horizontal pipes and the electrons can proceed unhindered vertically to oxygen. It is obvious from this model that a disturbance in respiration may occur at numerous points in this engine, and the closing of any valve, either in the chain of carbon transport or in the chain of electron transport, would have discernible effects on the over-all metabolic pattern of the cell.

From this model we can visualize three possible ways in which aerobic glycolysis would be stimulated. First, if there is a disturbance in electron transport, so that one of the valves in the vertical pipes is closed, electrons would pile up and would have no other choice than to combine with an electron acceptor such as pyruvic acid to form lactic acid. Many objections to this hypothesis could be offered, but probably the most serious one is that oxygen consumption is relatively normal in tumors; if the normal electron transport mechanism does not operate in tumors, it would be necessary to make the unlikely assumptions either that some other electron transfer mechanism operates, or possibly that substances other than carbohydrates and fatty acids provide the fuel for tumor metabolism. The second possibility is that one of the steps leading pyruvic acid into the citric acid cycle is defective. This theory gains credence from earlier work of Elliott and co-workers (5), who found pyruvate oxidation to be very low in tumors; and particularly from recent findings of Potter and LePage (10), who failed to obtain pyruvate or oxalacetate oxidation in fortified tumor homogenates. Any failure of pyruvate to undergo oxidation could conceivably result in its competing for electrons with other electron acceptors, thus leading to lactic acid formation. I will defer discussion of the third possibility until later.

The question with which we concerned ourselves in the present study was: Do tumors utilize glucose and fatty acids as substrates for oxidation and energy production? If so, do they utilize them to the same extent and in the same ways as normal tissues? Definite answers to these questions have not as yet been obtained by means of the usual methods of study of oxidative metabolism. A serious impediment to the study of oxidative processes is what may be termed the "metabolic iner-

tia" of isolated, surviving tissue slices. These have a rather high endogenous metabolism which is not changed materially by the addition of various metabolites, so that oxygen consumption data alone do not tell whether a particular metabolite is oxidized. Much progress has been made by studying oxidative processes in minces and homogenates, because in these preparations endogenous metabolites can be removed by dilution or washing. Unfortunately, disruption of tumor cells virtually eliminates all oxidative capacity. Therefore, most of our knowledge of tumor metabolism has been based on assays for individual enzymes which, when present, indicate what *can* happen, but not necessarily what *does* happen. The availability of isotopically labeled compounds has afforded us a simple, direct, and reliable method of establishing whether a particular foodstuff can be oxidized by tumor cells. When a C^{14}-labeled substance is added to slices of tumor, its oxidation is manifested unequivocally by the appearance of radioactivity in the respiratory carbon dioxide. Thus, by measurement of the amount and activity of the CO_2 it was possible to determine the oxidative capacity of tumor tissues for carbohydrates, fatty acids, and various of their intermediates. The experimental procedure is quite straightforward. Slices of tumor tissue[1] in amounts of 2–3 gm. fresh weight are placed in the Warburg flasks shown in Chart 2, which have a volume of approximately 125 ml. CO_2-free alkali is placed on filter paper held in a small glass cup which fits loosely in the center well. The flasks, containing the labeled substrate in concentrations of approximately 0.005 M, in a Ringer-phosphate medium, are oxygenated, attached to the manometers, and shaken for 2–3 hours at 38° C., after which process acid is tipped in from the side-bulb to liberate any CO_2 bound by the medium. CO_2 is then recovered from the filter paper and assayed by standard procedures.

RESULTS

Oxidation of isotopically labeled substrates.—Because most of the discussions concerning the oxidative metabolism of tumors have centered about carbohydrates, it was of considerable interest to compare the rates of glucose oxidation in normal and neoplastic tissue slices. Results of such experiments are given in Table 2. The term "O.C." is one we have coined to indicate the oxidative capacity of the tissue for the substance in question. It represents the micro-atoms of carbon of the substrate, in this instance glucose, converted to CO_2 per gram of dry tissue per hour. This value can be easily calculated from the amounts and relative specific activities of the respiratory carbon dioxide.

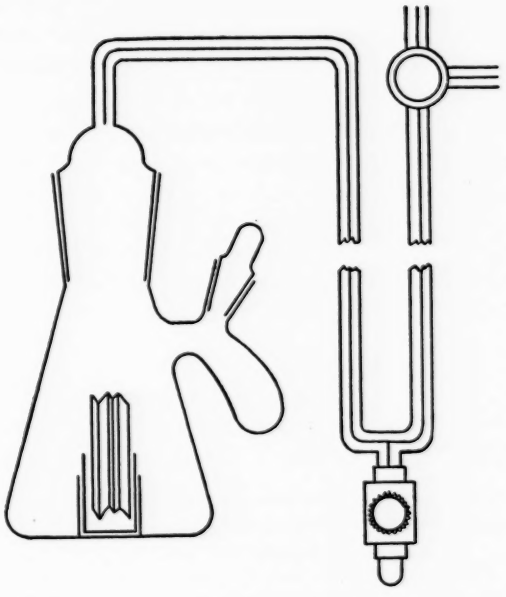


CHART 2.—Large-size Warburg flasks used for oxidation experiments with isotopic substrates.

TABLE 2

GLUCOSE OXIDATION BY NORMAL AND NEOPLASTIC TISSUE SLICES

Tissue	O.C.* Micro-atoms carbon
Normal, rat:	
Kidney	111
Brain (homogenate)	93
Heart	87
Liver	23
Skeletal muscle (homogenate)	7.0
Neoplastic:	
Hepatoma (mouse)	43.8
Hepatoma (rat)	38.3
Rhabdomyosarcoma (mouse)	35
Mammary adenocarcinoma (mouse)	25.5

* O.C. refers to the micro-atoms of substrate carbon oxidized to CO_2/gm dry tissue/hour at 38° at substrate concentration of 0.005 M.

The normal tissues fall into two groups: those with a high capacity for oxidizing glucose, such as kidney, brain, and heart; and those with a rather

[1] The tumors used in this study are all subcutaneous transplants carried for many generations and studied thoroughly in various departments of our Institute. The experimental work to be described was carried out by Mrs. Ruth Millington, Mr. Charles Wenner, and Dr. Morris Spirtes.

We are greatly indebted to the following individuals for invaluable aid in this work: Drs. Theodore Hauschka and Irene Diller for the mammary adenocarcinoma and much expert advice on the inbreeding of mice and transplantation methods; Dr. Grace Medes, for supervision of the animal colony and transplantation; Dr. Andrew J. Donnelly, for histological examinations; Dr. Julius White, for the mouse hepatoma; and Dr. W. F. Dunning, for the rat hepatoma. We also wish to thank Drs. Stern and Ochoa for details of the "condensing" enzyme assay.

low capacity—namely, liver and skeletal muscle. The four tumors have rates of glucose oxidation which are intermediate between the higher and lower normal tissues, and they exhibit a surprisingly narrow range of variation. This observation is in harmony with the views of Greenstein (1), Potter (9), and others, who have pointed out that tumors tend to have a similar metabolic pattern. Table 3 shows that the natural, 16-carbon acid, palmitic, is oxidized by all three tumors at rates about equal to its oxidation by normal liver and kidney.

The data in Table 4 on lactic acid oxidation are of particular interest because of the high aerobic glycolysis of neoplastic tissue. Here again we observe a wide range of variation in oxidative capacity between normal tissues, as compared to a relatively narrow range in the five tumors studied.

TABLE 3

PALMITATE* OXIDATION BY NORMAL AND NEOPLASTIC TISSUES

Tissue	Q.C.
Normal rat:	
Kidney	39.8
Liver (fasted)	18.6
Liver (fed)	10.0
Liver of tumor-bearing rat	8.7
Neoplastic:	
Hepatoma (mouse)	20.3
Hepatoma (rat)	13.6
Mammary adenocarcinoma (mouse)	11.0
Rhabdomyosarcoma (mouse)	16.5

* Substrate concentration = 0.001 M.

Some comparisons are noteworthy; hepatoma oxidizes lactic acid faster than liver, and rhabdomyosarcoma oxidizes lactate more rapidly than skeletal muscle. This latter comparison may be unfair, however, since it is difficult to maintain good respiration in minces of skeletal muscle. Other data are available for other substances such as short-chain fatty acids and for succinic acid; but the examples cited are sufficient to indicate that these tumors oxidize glucose and fatty acids about as well as do normal tissues.

The question which now emerges is whether these oxidations proceed by the same pathways in tumor as in normal tissues. A categorical answer would require a complete documentation of each step in the paths of carbon and electron transport, and this has not been done. However, again by means of the isotopic tracer method, definite evidence was obtained for the participation of citric acid as an intermediate; and this can be taken as rather conclusive evidence for the occurrence of the citric acid cycle, which is the mechanism utilized by normal tissues for the oxidation of carbohydrates and fatty acids. The procedure employed was to incubate the tumor slices with the labeled substrates in the presence of a large amount of normal, nonisotopic citric acid. We anticipated that if isotopic citrate were formed metabolically, a small amount of it might mix with the unlabeled citrate, be trapped thereby, and could then be detected by the presence of radioactivity in the citrate isolated and purified after recovery from the solution. The data shown in Table 5 are only of qualitative significance, because we do not have any way of determining to what extent the extracellular added citrate comes into equilibrium with the metabolic, intracellular citrate. It seems fair, however, to assume that such equilibration would be very slow; consequently, the low activities observed are not at all unexpected. Further significance is added to these results by comparing them with results of similar experiments with nor-

TABLE 4

LACTIC ACID* OXIDATION BY NORMAL AND TUMOR TISSUES

Tissue	Q.C.
Normal:	
Rat liver	44
" kidney	152
" heart	148
" muscle	3
" brain	112
" spleen	130
Mouse liver	32
" kidney	440
" heart	108
" muscle	5
Neoplastic:	
Rat hepatoma	61
Mouse hepatoma	87
" Andervont hepatoma	48
" Sarcoma 37	104
" rhabdomyosarcoma	72
" mammary adenocarcinoma	65
" Ehrlich ascites	116

* Carboxyl-labeled lactate, concentration = 0.0005 M.

TABLE 5

RADIOACTIVE CITRATE FROM TUMOR OXIDATIONS

Tissue	Substrate	Activity (c/m)	Quinidine citrate activity (c/m)
Normal, mouse:			
Heart	Glucose	5.5×10^5	150
Liver	"	"	126
Kidney	"	"	2,480
Neoplastic, mouse:			
Hepatoma	Glucose	1.37×10^6	1,225
Mammary tumor	"	"	1,750
Rhabdomyosarcoma	"	5.5×10^5	1,000
Ehrlich ascites	"	"	910
Mammary tumor	Acetate	1.4×10^6	293
"	Palmitate	66,200	74
Hepatoma	"	"	138
Rhabdomyosarcoma	"	"	83

mal tissues. Of the three tested, only kidney gave a citrate activity comparable to the tumors. The low uptake of activity in citrate by heart and liver is probably due to preferential oxidation of citrate, since oxidation of glucose was very low in these experiments in the presence of citrate.

Assay of tumors for enzymes of the citric acid cycle.—Further evidence for participation of the citric acid cycle in tumor oxidations has been obtained through assays of the tumors for individual enzymes of the citric acid cycle. Perhaps the most interesting of these, from the standpoint of tumor metabolism, is the "condensing" enzyme. This enzyme which has been studied, and recently obtained in crystalline form by Stern, Shapiro, and Ochoa (11), carries out the condensation of acetyl-CoA, a compound of acetate with the new co-

TABLE 6

CONDENSING ENZYME ASSAY

Tissue	Micromoles citrate/ 10 min/100 mg acetone powder at 25°
Mouse liver	1.53
Rhabdomyosarcoma	1.45
Hepatoma	2.90
Mammary tumor	3.30

enzyme, of which pantothenic acid is a component, to yield citrate and CoA, as shown in equation (1).

$$\text{Ac-CoA} + \text{Oxalacetate} \xrightarrow{\text{condensing enzyme}} \text{Citrate} + \text{Coenzyme A.} \quad (1)$$

Since it is impossible to obtain acetyl-CoA as such, it has to be synthesized *in situ*. This can be done by a factor present in an extract of lyophilized *E. coli*, which catalyses the transfer of acetyl from acetyl phosphate to coenzyme A (equation [2]).

$$\text{Acetyl phosphate} + \text{CoA} \xrightarrow{\textit{E. coli}\ \text{factor}} \text{Ac-CoA} + \text{phosphate.} \quad (2)$$

Thus, by adding acetyl phosphate, *E. coli* extract, coenzyme A, and oxalacetate to a tissue, the extent of citrate formation is a measure of its content of condensing enzyme. Some comparisons are given in Table 6.

The values are in micromoles of citrate produced per 10 minutes/100 mg of acetone powder. From the data presented the condensing enzyme is present in amounts as high as or higher than in normal mouse liver. Data for the three dehydrogenases, lactic, malic, and isocitric, are given in Table 7. While quantitative differences are evident between normal and tumor tissues, these are no greater than between different kinds of normal tissues. Of all the enzymes studied aconitase exhibited the greatest difference, being considerably lower in tumor than in normal tissues (Table 8). However, this enzyme is notably labile (2), and there is no certainty at present how much inactivation occurred during preparation. At any rate, aconitase is undoubtedly present in the tumors studied. Fumarase occurs in tumors in amounts comparable to those in normal tissues, and oxalacetic decarboxylase activity in tumors is of the same magnitude as in normal tissues.

TABLE 7

DEHYDROGENASE ASSAYS IN ACETONE POWDER EXTRACTS*

Tissue	Units†/mg acetone powder		
	Lactic	Malic	Isocitric
Normal:			
Heart (rat)	320	383	56.0
Liver (mouse)	200	256	10.8
Kidney (rat)	104	173	66.0
Muscle (mouse)	520	330	15.6
Neoplastic:			
Rhabdomyosarcoma (mouse)	160, 220	148, 208	6.7
Mammary tumor (mouse)	168	380	16.0
Hepatoma (rat)	238	540	12.5–12.8
Hepatoma (mouse)	108	288	14.8
Ascites	165	200	4.8

* Acetone powders prepared from pooled tumors.

† Each unit represents a change in optical density of 0.01 per minute at 24°–26° at 340 mμ.

TABLE 8

ASSAYS OF ACONITASE, FUMARASE, AND OXALACETIC DECARBOXYLASE

Tissue	Aconitase Units/mg dry wt of tissue*	Fumarase Units/mg dry wt of acetone powder*	Oxalacetic Decarboxylase Q_{CO_2}
Normal:			
Liver (mouse)	33	132	3.21
Heart (rat)	72	96	
Kidney (rat)	50	62	
Liver (rat)	14–25	66	4.70
Muscle (rat)	12		
Neoplastic:			
Rhabdomyosarcoma (mouse)	2.2–5.0	50.2	2.17
Hepatoma (mouse)	7.0–8.3	61.5	1.26
Mammary adenocarcinoma (mouse)	3.1–5.9	49.5	
Hepatoma (rat)	4.9	133	2.45
Ehrlich ascites			3.48

* A unit represents a change in optical density of 0.001 per minute at 25° at 240 mμ.

Oxidation processes in tumor homogenates.—From the data thus far presented there appears to be no justification for assuming there is any fundamental disturbance in carbohydrate or fatty acid metabolism in tumors. Glucose and palmitic acid, both natural foodstuffs, are oxidized as readily in tumor as in normal cells; and the high rate of lactic acid oxidation displayed by tumors is incom-

patible with the belief that the high aerobic glycolysis is due to inability of the tumors to oxidize lactic acid. Accordingly, the question of why pyruvate and oxalacetate are not oxidized in tumor homogenates (10) assumes renewed significance. It seemed to us that lack of oxidative capacity of tumor homogenates might be due to destruction of some labile factor during homogenization. Some support for this viewpoint is found in the fact that glycolysis in both normal and tumor tissues is also inhibited by homogenization, but can be restored by the addition of various co-factors (1, 7, 8).

TABLE 9

EFFECT OF DPN LEVEL ON FUMARATE OXIDATION IN WASHED MOUSE HEPATOMA RESIDUE

The medium contained the following: tumor homogenate, equivalent to 50 mg. of dry tissue; fumarate, 0.005 M; phosphate, pH 7.4, 0.016 M; ATP, 0.002 M; cytochrome c, 0.1 mg.; Mg SO_4, 0.003 M; yellow enzyme, 0.1 ml. in a total volume of 1.6 ml. Experiments run 1 hour at 38° in air.

Added DPN final concentration	$\mu l. O_2$
0	5.4
4×10^{-5}	9.5
4×10^{-4}	25.1
1.5×10^{-3}	77.0, 69.0

TABLE 10

OXIDATION OF CITRIC ACID CYCLE SUBSTRATES BY WASHED MOUSE HEPATOMA RESIDUE

Conditions essentially the same as Table 9; final DPN concentration, 0.0015 M.

Substrate	$\mu l.O_2$
0	22.6
Pyruvate	58.8
α-Ketoglutarate	82.7
Citrate	136

The correctness of this assumption was borne out when it was found that pyruvate, oxalacetate, and other components of the citric acid cycle could be oxidized by tumor homogenates if these were strongly fortified with diphosphopyridine nucleotide. The results thus far obtained are regarded as preliminary, but there seems to be no doubt that oxygen consumption can occur even in well washed subcellular particles. Table 9 shows the effect of increasing concentrations of DPN, and Table 10 gives data for several citric acid cycle components with optimal fortification with DPN. Table 11 gives data for oxalacetate oxidation by whole homogenates of four different tumors. DPN is the only co-factor having a consistent activating effect; occasionally, activation was achieved with a crude preparation of flavin adenine nucleotide, but few or no effects were found with cytochrome c, triphosphopyridine nucleotide, cocarboxylase, or adenine nucleotides.

In conclusion, we may again consider the factors concerned in the high glycolysis of tumors; and to do this let us again refer to the cellular engine in Chart 1. From the results already presented it is fairly certain that all the horizontal and vertical valves are open and functioning normally in tumors; and this leaves us with the only alternative of assuming that in tumors the rate of flow of carbohydrate carbon atoms is very rapid—so rapid that the *normal* capacities of the valves and pipes are exceeded. Lactic acid accumulation, it seems, results from "backing up" of electrons and pyruvate, because neither the electron transport enzymes nor the enzymes for pyruvate oxidation can cope with the rapid formation of their substrates. On the basis of the available evidence, this

TABLE 11

OXYGEN CONSUMPTION IN WHOLE TUMOR HOMOGENATES

Conditions same as Table 9; final DPN concentration, 0.0015 M.

	Oxygen consumptions, $\mu l.$ Additions			
	None	DPN	OAA	DPN+OAA
Hepatoma (mouse)	45	152	28	252
" (rat)	40	133	44	118
Mammary tumor	33	142	48	136
Rhabdomyosarcoma	63	168	66	167

seems to represent the most reasonable explanation for the phenomenon of high aerobic glycolysis.

Since high rates of glycolysis are a characteristic feature of growing tissues (whether malignant or not), one is led to the conviction that this high glycolytic rate is simply a result or a manifestation of the rapid growth. Although relationships between growth and glycolysis cannot yet be stated in chemical equations, there seems to be good reason to believe that these processes would be highly integrated. Several obvious common factors in the two processes are the nucleotides, ATP and DPN. The parts these substances play in glycolysis are too well known to require further comment. It is becoming increasingly evident that the biosynthetic reactions, which both Drs. Potter and Zamecnik are discussing, utilize the high-energy phosphate bonds of ATP for their energy requirements. On these grounds it seems plausible that rapid growth would be associated with changes in

the intracellular proportions of ATP, ADP, and phosphate, and such changes would most certainly lead to profound alterations in glycolytic rates.

It is also most probably true that many of these synthetic reactions which are reductive processes (amination, fatty acid synthesis, etc.) are coupled with oxidative processes through common coenzymes. Rapid growth can therefore be presumed to alter the ratio of reduced to oxidized DPN; and since these different forms of the nucleotide have different affinities for the apoenzymes, a small change in their ratio could conceivably lead to great changes in the over-all rate of such a DPN-dependent process as glycolysis.

It is hoped that experimental appraisal of these concepts will clarify the relationship of glycolysis to protoplasmic synthesis. At any rate, whatever may be the cause of the high aerobic glycolysis in tumors, it seems definite now that it is not due to quantitative or qualitative peculiarities of oxidative metabolism.

REFERENCES

1. BOYLAND, E., and BOYLAND, M. E. Studies in Tissue Metabolism. VII. The Action of Tumor Extracts on Hexose Diphosphate. Biochem. J., **29**:1910, 1935.
2. BUCHANAN, J. M., and ANFINSEN, C. B. Partial Purification of Aconitase. J. Biol. Chem., **180**:47, 1949.
3. BURK, D. A Colloquial Consideration of the Pasteur and Neo-Pasteur Effects. Cold Spring Harbor Symp. Quant. Biol., **7**:420, 1939.
4. DICKENS, F., and SIMER, F. The Respiratory Quotient and the Relationship of Respiration to Glycolysis. Biochem. J., **24**:1301, 1930.
5. ELLIOTT, K. A. C.; BENOY, M. P.; and BAKER, Z. The Metabolism of Lactic and Pyruvic Acids in Normal and Tumor Tissues. II. Rat Kidney and Transplantable Tumors. Biochem. J., **29**:1937, 1935.
6. GREENSTEIN, J. P. Biochemistry of Cancer. New York: Academic Press, Inc., 1947.
7. MEYERHOF, O., and WILSON, J. R. Studies on the Enzymatic System of Tumor Glycolysis. I. Glycolysis of Free Sugar in Homogenates and Extracts of Transplanted Rat Sarcoma. II. Comparative Study of Rat and Mouse Tumor Homogenates. Arch. Biochem., **21**:1, 22, 1949.
8. NOVIKOFF, A. B.; POTTER, V. R.; and LEPAGE, G. A. Phosphorylated Intermediates in Tumor Glycolysis. IV. Glycolysis in Tumor Homogenates. Cancer Research, **8**: 203, 1948.
9. POTTER, V. R. Biological Energy Transformations and the Cancer Problem. Advances in Enzymology, **4**:201, 1944.
10. POTTER, V. R., and LEPAGE, G. A. Metabolism of Oxalacetate in Glycolyzing Tumor Homogenates. J. Biol. Chem., **177**:237–45, 1949.
11. STERN, J. R.; SHAPIRO, B.; and OCHOA, S. Synthesis and Breakdown of Citric Acid with Crystalline Condensing Enzyme. Nature, **166**:403, 1950.
12. STERN, K., and WILLHEIM, R. The Biochemistry of Malignant Tumors. Brooklyn: Reference Press, 1943.
13. WARBURG, O. Metabolism of Tumours. London: Constable & Co., 1930.

Paper No. 4 in the Symposium on Intermediary Carbohydrate Metabolism in Tumor Tissue

Studies on the Carbohydrate and Protein Metabolism of the Rat Hepatoma*

PAUL C. ZAMECNIK, ROBERT B. LOFTFIELD, MARY L. STEPHENSON, AND JEAN M. STEELE

(Medical Laboratories of the Collis P. Huntington Memorial Hospital of Harvard University, Massachusetts General Hospital, Boston, Mass.)

The availability of carbon 14-labeled glucose has made it possible to re-investigate and extend Warburg's (34) finding of an increased aerobic glycolytic rate in tumors. While this metabolic property of neoplasms to degrade glucose to lactic acid at an accelerated rate is shared by certain normal tissues, it remains perhaps the nearest approach to a biochemical peculiarity of tumor tissue.

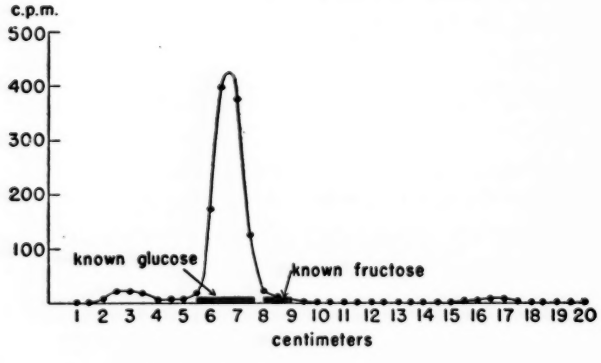


CHART 1.—Peaks of radioactivity found in glucose-C^{14} prepared from purified starch; 10 c.mm. of a solution containing 2,000 c.p.m. in approximately 20 μg. of glucose was chromatographed. This type of preparation was used in the early experiments.

Although glucose has been labeled by direct organic chemical methods (11, 15, 28) in a single position, it appeared advantageous to employ glucose labeled in all 6 carbons for a study of the fate of glucose fragments in the flow of the various metabolic currents within the cell.

* This is publication 738 of the Harvard Cancer Commission. This work was supported by grants-in-aid from the American Cancer Society and the Atomic Energy Commission.

METHODS

PREPARATION OF C^{14}-LABELED GLUCOSE[1]

C^{14}-labeled glucose was prepared from bean leaves according to either the method of Villee and Hastings (33) or a combination of the methods of Putnam *et al.* (25) and Pucher *et al.* (24). It was found by paper chromatography (21–23) that a small percentage of a radioactive contaminant was present in the product obtained by either method, in our hands (Chart 1). Further purification was achieved by a chromatographic method similar to that described by Udenfriend and Gibbs (31).

The yields of glucose-C^{14} obtained from starch were only a few per cent of the $BaC^{14}O_3$ added. In order to improve the total yield and to obtain fructose-C^{14}, the 80 per cent alcoholic extract of bean leaves was treated according to Putnam *et al.* (25) (Chart 2), except that paper chromatographic purification was substituted for crystallizations. The procedure used differed from that of Udenfriend and Gibbs (31) in that a single strip of relatively thick filter paper was used for chromatography rather than a number of thin strips.

Quantities of material up to 30 mg. were dissolved in a minimum of water and were pipetted along a 10-cm. horizontal line near the top of a sheet of Whatman No. 3 filter paper, 50 cm. in length. Ten pipettings from a 10-c.mm. pipette were used to keep the solute line a thin one, covering a minimum band width. Descending chromatograms were run, with butanol-acetic acid (21). Chromatograms were run for approximately 48 hours, the solvent front being allowed to drip

[1] $BaC^{14}O_3$ and some of the glucose used in these experiments were supplied by the Isotopes Division, U.S. Atomic Energy Comm., Oak Ridge, Tenn. We are especially indebted to Dr. Martin Gibbs for generous samples of glucose-C^{14}.

off the paper for at least 12 hours. A strip of paper a few mm. wide was cut off each side, and a similar strip was cut down through the middle of the filter paper. These strips were then sprayed with ammoniacal silver nitrate (21), to locate the positions of glucose and fructose. Known samples of glucose, fructose, and sucrose were also chromatographed on adjacent paper strips for identification of position. Location of the bands was also outlined by fluorescence, using a Mineralight lamp, in order to facilitate the cutting.

A strip of the chromatogram 2 mm. in width was cut off and divided lengthwise into 1-cm. segments, which were mounted consecutively on brass discs and assayed for radioactivity. In this way the location of a glucose band, free from fructose and sucrose, was possible. The horizontal band containing the glucose was then cut from the rest of the chromatogram. Some inert paper was sewed onto this active piece, dipped into a trough of water, and the glucose was eluted by dripping off as a descending chromatogram from the lower end of the paper strip, to which a piece of inert paper terminating in a point had been sewn. The eluate dripped into a small funnel inserted in a test tube. Two cc. of water has been sufficient to elute an active piece of paper 10×8 cm. in size. This aqueous solution of glucose also contained acetic acid and butanol from the original chromatograph. The solution was lyophilized to dryness, and the glucose was taken up in water. We have rechromatographed this preparation of glucose, using two different solvent systems (phenol-water, and butanol-water), and have found only one peak of radioactivity (Chart 3). A chromatographic purification method has also recently been described by Noggle and Bolomey (20).

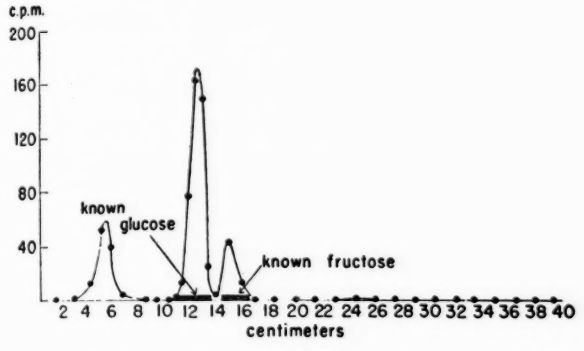


CHART 2.—Peaks of radioactivity found in a glucose preparation obtained by crystallizing glucose-C^{14} out of an alcoholic extract of bean leaves. The unlabeled contaminant is probably sucrose, since a parallel chromatogram showed inert sucrose to move with the same RF value, and a sample of known sucrose-C^{14} gave a single peak in this same region.

SYNTHESIS OF LABELED COMPOUNDS

The DL-alanine-1-C^{14} was prepared by conventional methods (13), and no particular difficulties were encountered.

Calcium lactate-1-C^{14} was synthesized by hydrolysis of acetaldehyde cyanhydrin (6).

Pyruvic acid-2-C^{14}, however, presented a problem. When acetyl cyanide was hydrolyzed by the method of Tschelinzeff and Schmidt (30) or of Calvin and Lemmon (5), by far the largest part of the radioactivity appeared in acetate. If acetyl cyanide was hydrolyzed to the amide (2) and the amide hydrolyzed with hydrochloric acid, only 60 per cent of the radioactivity appeared to be in the form of pyruvic acid, although a larger fraction could be isolated as pyruvic acid dinitrophenylhydrazone. Presumably this is accounted for by the partially reversible aldolization reactions of pyruvate. The best method for obtaining "pure" pyruvate consisted in adding a saturated ethanolic solution of sodium acetate to an ethanolic solution of pyruvic acid-2-C^{14}. The sodium pyruvate which precipitated contained 85–90 per cent of the radio-

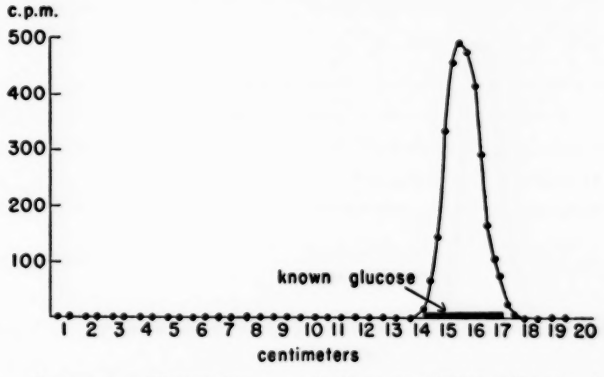


CHART 3.—Chromatogram of glucose solution after purification on thick paper. A solution of glucose which had been purified as described in the text was pipetted in a spot on a strip of Whatman No. 1 paper; 10 c.mm. contained approximately 12,000 c.p.m. in 30 μg. of glucose. This chromatographically pure preparation was used in the later experiments.

activity in a single peak in a paper chromatogram, run according to Magasanik and Umbarger (14). This peak corresponded to authentic sodium pyruvate. The sodium pyruvate-C^{14} appeared to be stable in the dry state, but the great instability of pyruvates is indicated by the fact that after 3 months in a frozen solution, paper chromatography showed that only 20 per cent of the pyruvate was unchanged, and three new peaks containing 8, 50, and 20 per cent of the radioactivity had appeared.

RECOVERY OF $C^{14}O_2$

After incubation, 0.5-cc. aliquots of the gas phase were withdrawn into a 1-cc. greased tuber-

culin syringe. The air was introduced into a closed 60-cc. flask containing 5 cc. of 5 N NaOH (CO_2-free) and 0.1 mM Na_2CO_3. This was shaken vigorously, allowed to stand overnight, and then excess $BaCl_2$ was added. The NaOH was almost neutralized, using HCl. All the precipitate was filtered and mounted on brass discs for counting (10). This contained 0.1 mM of inert $BaCO_3$ plus a negligible amount of $C^{14}O_2$. The total radioactivity present in the CO_2 in the gas phase of the flask was calculated from the counts obtained from the 0.5-cc. aliquot and the total volume of the gas. In order not to interfere with the usual washing and processing of the slices after incubation, acid was not tipped into the incubation medium to drive out dissolved CO_2, except in an occasional instance. We have therefore expressed results as counts per minute in the gaseous CO_2 in the vessels.

Experimental Conditions

The medium used was that of Anfinsen *et al.* (1), containing the following ions expressed in mM/l: Ca^{++}, 10; Na^+, 32; K^+, 73; Cl^-, 85; HCO_3^-, 40. Alanine and sodium pyruvate used by Anfinsen were omitted. The pH was 7.4. Incubation was for 2 hours at 37° C. in an atmosphere of 95 per cent oxygen, 5 per cent carbon dioxide.

Experiments were carried out either in Warburg vessels or in flat-bottomed tubes, 2.5 cm. × 9.5 cm., stoppered with rubber vial caps. Each flask usually contained 1 cc. of medium, two or three slices of liver, 0.1 cc. of glucose-, pyruvate-, lactate-, or fructose-C^{14} solution (about 60,000 c.p.m.), and 0.1 cc. of solution containing additional substrates.

Hepatomas were produced, as previously described (37), in young female Harvard colony rats. In most experiments the rats were starved 3–6 days prior to use.

Treatment of Slices after Incubation

After incubation, tissue slices were first removed from the medium and washed in approximately 3 cc. of water, which was added to the medium. The slices were then washed with 2 20-cc. portions of water. They were homogenized in 5 cc. of water in a Potter-Elvehjem homogenizer and the proteins precipitated by addition of 5 cc. of 8 per cent trichloroacetic acid. The precipitate was washed twice with 10 cc. of 4 per cent trichloroacetic acid, once with 10 cc. of hot 4 per cent trichloracetic acid (90° C. for 20 minutes), once with 10 cc. of warm absolute alcohol, once with 10 cc. warm alcohol:ether:chloroform (2:2:1), and once with 10 cc. of warm acetone. The latter three washings were pooled and were considered the lipid fraction.

After each experiment, the medium was quantitatively removed from the flasks, made to 10 cc. with water, and was frozen.

Preparation of Samples for Counting

Protein.—The washed protein was dried by heating the tube slowly to boiling in a bath or by letting the tube stand at room temperature overnight. The dry protein was homogenized in acetone and plated on to filter paper (Whatman No. 50) in an apparatus similar to the one described by Melchior and Tarver (16). The area of the samples was 3.8 $cm.^2$ The protein was scraped off the hard filter paper after counting and was weighed. Self-absorption corrections were made for thickness of the protein sample, with a correction curve constructed by Siekevitz (27). A total of 1,024 or 2,048 counts was collected, and the statistical fluctuation due to counting was approximately 5 per cent. The over-all reproducibility of the counting procedures was as previously described (37).

$BaCO_3$ precipitates obtained by ninhydrin decarboxylation of protein hydrolysates.—In a few experiments the washed protein was hydrolyzed overnight in 1 cc. of 6 N HCl in an autoclave. The ninhydrin procedure was used to split off the α-carboxyl groups of amino acids. The carbon dioxide liberated was trapped in barium hydroxide, and the barium carbonate formed was filtered and counted (37).

Lipids.—The hot alcohol, hot alcohol:ether:chloroform, and hot acetone washings were combined. An aliquot was put in a stainless steel or nickel dish, dried in air, and was counted. No attempt was made to correct for the self absorption of the lipids, as the amount in the dishes was negligible in most cases.

Miscellaneous Methods

All counts obtained, unless otherwise stated, have been corrected to zero thickness.

Glucose concentration of the media was determined by using Nelson's adaptation of the Somogyi method (19).

The total radioactivity in glucose was determined by adding 0.20 gm. of inert glucose to an aliquot of the incubation medium followed by preparation, crystallization, and radioassay of the glucosazone. The value obtained was compared with the figure obtained as a result of processing a known amount of glucose-C^{14}, under the same conditions.

Lactate determinations were carried out according to the method of Barker and Summerson (3), as modified in Umbreit (32).

Keto acids were determined as dinitrophenylhydrazones (8).

Glycogen was measured according to Good,

Kramer, and Somogyi (9), and fructose according to the method of Roe (26).

RESULTS

In vitro *results*.—In Table 1 are summarized the results of an experiment in which control and neoplastic hepatic tissue slices have been incubated with glucose-C^{14} in the presence and absence of oxygen. The radioactivity in the protein fraction of the slices was determined by ninhydrin decarboxylation of the protein hydrolysate and by the simpler procedure of counting the washed protein directly. The ninhydrin method served as a check on the question of whether the radioactivity associated with the protein is really located in amino acids.

The greater incorporation into the hepatoma protein is evident. This has been mentioned previously (38). A slight amount of incorporation occurs anaerobically, in agreement with findings of Winzler, Krause, and Slater (35), and here again it is greater in the hepatoma protein. The implication that radioactivity is located in positions other than the carboxyl position of amino acids is discussed at greater length later.

ber of counts, in the form of glucose-C^{14}, were added to each flask, but were diluted with varying amounts of inert glucose. From these results it appears that at all concentrations of glucose used the conversion to protein carbon is greater in the hepatoma slice. There is a suggestion, however, that in this conversion process the hepatoma slice operates at nearer its maximal rate at the lowest glucose concentration than does the normal liver slice. The

TABLE 1

INCORPORATION OF C^{14} FROM LABELED GLUCOSE INTO LIVER SLICE PROTEIN

	Control liver		Hepatoma	
	Oxygen	Nitrogen	Oxygen	Nitrogen
c.p.m. / 12 mg. protein	588	12	6,250	99
c.p.m. / 0.1 mM $BaCO_3$	148	2.1	2,040	9.6

The experimental conditions are described in the text. Each flask contained 0.44 mg. glucose-C^{14} containing 260,000 c.p.m. This represents a specific activity of approximately 0.4 mc/mM. In all the tables the figures represent the average of duplicate determinations. Rats were not starved. 0.1 mM $BaCO_3$ represents approximately 12 mg. of protein. The activity in each case has been corrected to zero thickness, as stated in the text.

TABLE 2

EFFECT OF GLUCOSE CONCENTRATION ON THE CONVERSION OF GLUCOSE CARBON TO PROTEIN CARBON

	Control			Hepatoma		
	Glucose concentration			Glucose concentration		
	0.0007 M	0.006 M	0.028 M	0.0007 M	0.006 M	0.028 M
C.p.m. per milligram protein	3.6	3.6	1.6	104	60	18.6
Per cent glucose converted to protein	0.07	0.06	0.03	2.2	1.1	0.21
Per cent protein carbon derived from glucose carbon	0.0004	0.003	0.006	0.01	0.05	0.05
Mg. lactic acid per flask	0	0.04	0	0.24	0.62	0.81

Experimental conditions described in text.
Each flask contained 100,000 c.p.m. in glucose.
Rats were starved 6 days.

The question arises whether the above-mentioned results may be artifacts, due to a greater dilution of glucose-C^{14} in the normal hepatic slice by inert glucose derived from glycogen stored in the slice. Whereas the liver glycogen level was found to be 2.1, 2.7, and 4.4 per cent of the wet weight in three normal, unstarved rat livers, it was found to be 0.04, 0.09, and 0.15 per cent in three hepatomas. Rats were therefore starved for varying lengths of time, and it was determined that after 6 days' starvation no glycogen could be detected in either normal liver or hepatoma. Slice experiments were carried out on such rats, and the differences previously observed on increased conversion of glucose-C^{14} into C^{14}-labeled protein in the hepatoma slice were confirmed, as illustrated in Table 2. The increased lactic acid production in the hepatoma slice is also shown. The same num-

possibility that differences in dilution by endogenous metabolites may be responsible for some of the apparent rate changes must also be considered.

To test the reproducibility of this metabolic finding, a series of nine experiments have been carried out embracing various states of the animal prior to the slice experiment—starvation, hypophysectomy, and alloxan diabetes, plus variations in glucose concentration and incubation time. Under these diverse circumstances (Table 3), the conversion of C^{14} from glucose to protein, CO_2, and lipid was in all cases greater in the hepatoma slice than in the normal liver slice (the ratios are given as H/N values).

In making this type of metabolic comparison, it would be useful to include many types of normal tissues rather than a single one. Among the normal tissues sharing with neoplastic tissue a high rate

of aerobic glycolysis are kidney medulla and brain. Table 4 shows that the conversion of glucose-C^{14} to protein-C^{14} in the hepatoma slice exceeds that in these two tissues, although the rate of disappearance of glucose was in the same range as that of the hepatoma. The kidney medulla behaves much more like the hepatoma in conversion of glucose carbon to protein than does the brain slice.

TABLE 3

INCUBATION OF LIVER SLICES IN GLUCOSE-C^{14}

Date of exper.	Protein*			Carbon Dioxide†			Lipid†		
	Normal	Hepatoma	H/N	Normal	Hepatoma	H/N	Normal	Hepatoma	H/N
11-1-49	47	507	11						
11-7-49	49	520	10						
11-29-49	3.6	105	28						
4-13-50	0.55	22	41						
1-16-50	56	2,560	46						
1-26-51	5.5	91	16	400	1,400	3.6	116	304	2.6
2-16-51	6.9	57	8	350	1,580	4.5	38	181	4.8
2-17-51	2.8	49	17				21	210	10
3-8-51	7.5	85	11	691	5,400	7.8	63	428	6.8

11-1-49. 260,000 c.p.m. in 0.44 mg. glucose. Rats not starved.
11-7-49. " " " " " " " "
11-29-49. 100,000 c.p.m. in 0.13 mg. glucose. Rats starved 6 days.
4-13-50. 17,000 c.p.m. in 0.1 mg. glucose. 30-minute incubation. Rats starved 5 days.
1-16-50 1,255,000 c.p.m. in 1 mg. glucose. Rats starved 4 days.
1-26-51. 60,000 c.p.m. in 0.02 mg. glucose. Rats not starved.
2-16-51. " " " " " " " "
2-17-51. " " " " " Alloxan diabetic rats, not starved.
Nonmalignant rat blood sugar, 380 mg/100 cc blood, hepatoma rat blood sugar, 408 mg/100 cc blood.
3-8-51. Hypophysectomized rat with hepatoma, nonhypophysectomized control rat. Rats not fasted. 60,000 c.p.m. in 0.02 mg. glucose.

* Cpm/mg protein.

† Cpm/100 mg wet tissue. Total $C^{14}O_2$ produced per 100 milligrams wet tissue would be roughly 3 times that found in the gas phase in experiments 1-26-51 and 2-16-51, and roughly 1.5 times that found in experiment 3-8-51, because of differences in flask volume.

TABLE 4

CONVERSION OF GLUCOSE-C^{14} TO PROTEIN-C^{14} IN RAT TISSUES

	C.p.m. per milligram protein	Per cent of glucose-C^{14} disappeared
Hepatoma	20.4	79
	24.7	84
Control-hepatoma	0.60	49
Normal liver	0.54	36
	0.56	36
Kidney medulla from normal rat	5.3	51
	6.3	43
Brain from normal rat	0.68	73
	0.39	61

Each flask contained 0.1 mg. glucose-C^{14}, containing 16,800 c.p.m. 30 minute incubation. Rats were starved for 5 days. Glycogen was not determined in this experiment. Control-hepatoma is the designation for the nonmalignant or control part of a hepatoma-containing liver.

In Chart 4 is shown a comparison of the rate of $C^{14}O_2$ production from glucose in the normal and neoplastic liver slice. We have consistently found a greater rate of degradation to $C^{14}O_2$ in the neoplastic liver slice.

In previous experiments (7, 36) we have found, in agreement with Anfinsen *et al.* (1), that, if tissue slices are incubated in a medium containing $NaHC^{14}O_3$, C^{14} becomes fixed into proteins. The question arises whether the glucose-C^{14} is degraded all the way to $C^{14}O_2$ in the above-mentioned series of experiments, or whether it may be converted by way of 2–3 carbon fragments. One experiment bearing on this point is summarized in Table 5. It appears unlikely from these data that the glucose molecule is degraded all the way to $C^{14}O_2$ before resynthesis to hepatoma protein, although the evidence is not so persuasive in the normal slice. It is also true that $C^{14}O_2$ added to a slice from the outside may behave differently from $C^{14}O_2$ produced within the cell.

In order to locate the radioactivity in the proteins, washed protein from a hepatoma slice and

DEGRADATION OF C^{14}-GLUCOSE TO $C^{14}O_2$

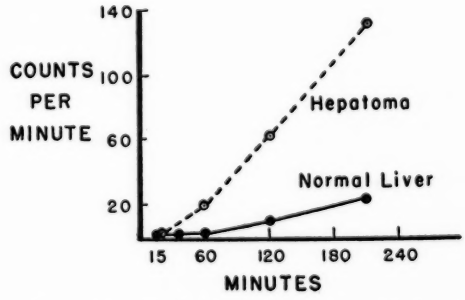


CHART 4.—Each flask contained 340,000 c.p.m. in 0.84 mg. of glucose-C^{14}, 2 cc. of medium, and either 4 slices of normal liver or 6 slices of hepatoma. Rats were starved 5 days. 0.4 cc. of the gas phase was withdrawn at 15, 30, 60, 120, and 210 minutes from the start of the experiment. The CO_2 was trapped and counted as described in the text. The radioactivity is recorded as cpm/mg wet liver at zero thickness. As an index of total $C^{14}O_2$ produced, the later hepatoma values may be as much as 15 per cent high, due to displacement of CO_2 from the medium by the lactic acid being produced.

from a normal liver slice was hydrolyzed, and starch column chromatograms were run (17, 18, 29, 36). The results are shown in Charts 5–7. In the hepatoma protein, radioactivity was located in the peaks identified with glutamic and aspartic acids, alanine, glycine, serine, and proline; and also with the leucine-isoleucine and methionine-valine peaks. Separation of glutamic acid from alanine required separate chromatographing under different conditions (Chart 6). The total counts in the normal liver protein were so low that it can only be stated with confidence that radioactivity was found in glutamic and aspartic acids, and in alanine. The solvent system in this normal liver protein fractionation was chosen so as to separate glutamic acid from alanine on the first run.

To probe this question a little more deeply, the alanine, glutamic, and aspartic acid peaks from the hepatoma chromatogram were subjected to further study. Unfortunately, the same type of study was not possible on the corresponding peaks from the normal liver chromatogram, since too little radioactivity was present with which to work. Furthermore, a sizeable fraction of the total

TABLE 5

COMPARISON OF GLUCOSE-C^{14} AND $C^{14}O_2$ AS SOURCES FOR INCORPORATION OF CARBON INTO LIVER PROTEIN

	Control		Hepatoma	
	CO_2	Glucose	CO_2	Glucose
Protein c.p.m. per 0.1 mM $BaCO_3$	28	35	17	864

This table represents two fairly typical experiments. In each experiment 100,000 c.p.m. were added to each flask, in one case as 0.002 mM of glucose-C^{14} and the other as 0.01 mM of $NaHC^{14}O_3$. One cc. of Anfinsen's medium without pyruvate or alanine in each flask. In both cases, flask contents were buffered with 0.04 M bicarbonate, with 5 per cent CO_2 in the gas phase. The counts tabulated are obtained from ninhydrin decarboxylation of the washed, hydrolyzed protein. Experiments on CO_2 fixation in normal and malignant hepatic slices have previously been described (36, 7). Counts are recorded at 0.1 mM thickness of $BaCO_3$.

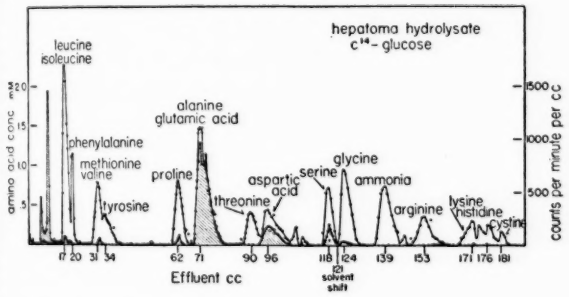


CHART 5.—Separation of amino acids in hepatoma protein hydrolysate. Three hepatoma slices were incubated as described in text, in a medium to which was added 1 mg. of glucose-C^{14} containing 1,250,000 c.p.m. The hepatoma protein hydrolysate added to the 1.1 cm. column contained 11,600 c.p.m. in 40 μM of amino acids. The initial solvent system was 1:2:1 *n*-butanol, *n*-propanol, 0.1 N HCl, and was shifted to 2:1 propanol:0.5 N HCl, at 120 cc. The effluent contained sufficient radioactivity to permit the collection of 0.2 cc. fractions on copper planchets ringed with beeswax, which were used directly for counting. Alternate fractions were collected in test tubes for colorimetric ninhydrins. The high peaks of radioactivity emerging prior to the leucine-isoleucine peaks are unexplained. The shaded areas represent radioactivity.

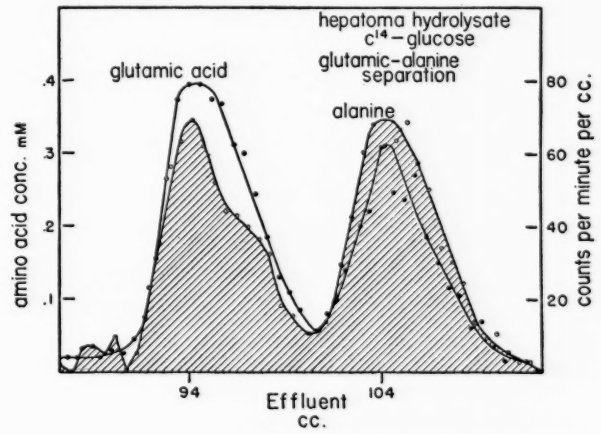


CHART 6.—Separation of the glutamic acid-alanine peak. An aliquot of the original hepatoma protein hydrolysate (0.08 cc. containing 150 c.p.m.) was taken up in 0.5 cc. 2:1:1 tertiary butanol, secondary butanol, 0.1 N HCl, to which 4 μM of glutamic acid, 1 μM of alanine, and 1 μM of proline had been added as carrier for identification purposes; 0.2-cc. fractions were collected and assayed as described in Chart 5.

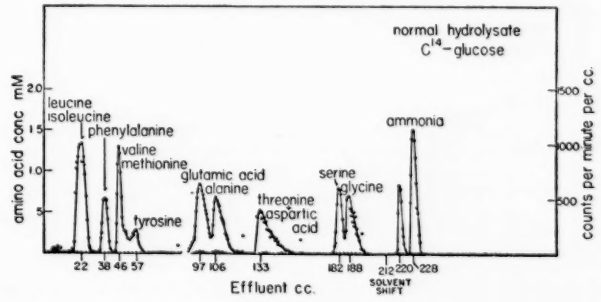


CHART 7.—Separation of amino acids in normal liver protein hydrolysate. Two normal liver slices were incubated as described in text, in a medium to which was added 1 mg. of glucose-C^{14} containing 1,250,000 c.p.m. The protein hydrolysate added to the 1.1 cm. diameter column contained 370 c.p.m. The solvent system was 2:1:1 tertiary butanol, secondary butanol, 0.1 N HCl. The solvent was shifted to 2:1 propanol: 0.5 N HCl after 212 cc. had been collected. Unfortunately the colorimetric ninhydrin determination was a failure beyond the glycine peak because of a yellow color which appeared in the tubes. This we believe was due to corrosion by the solvent of a small piece of metal we had provided to prevent the drops from splashing on the copper planchets. No planchets contained any counts in this part of the chromatogram, however. A comparison of the total counts found in the three largest peaks in the chromatograms is as follows:

	Hepatoma	Control
Alanine	3,682	129
Glutamic acid	3,318	220
Aspartic acid	1,420	86

supply of glucose-C^{14} had been expended to carry this single chromatographic experiment up to this point.

The results, in the case of the hepatoma protein, are shown in Table 6. The total combustions reveal that more activity is present than can be accounted for in the carboxyl positions of the molecules, in which positions the radioactivity was determined by the ninhydrin procedure. This is in contrast to the situation in $C^{14}O_2$ fixation experiments (36), in which the radioactivity in aspartic acid was all accountable in the carboxyl positions.

TABLE 6

LOCATION OF RADIOACTIVITY IN AMINO ACIDS IN GLUCOSE INCORPORATION EXPERIMENTS

	Counts/min/cc	
	Ninhydrin	Total carbon
Alanine	23	59
Glutamic acid	16	44
Aspartic acid	468	1,200

Aliquots were taken for ninhydrin and total combustion determinations from pooled amino acid fractions from the glucose-C^{14}-hepatoma chromatogram (Chart 5). Enough inert amino acid was added to give a 0.1 mM $BaCO_3$ sample for counting. Results are expressed as total cpm/cc solution at 0.1 mM thickness.

the H/N ratio for conversion to protein is not so great as is the case for glucose-C^{14}. It is questionable whether there is any difference in rate of degradation to $C^{14}O_2$, and, in a single experiment, no difference in rate of conversion to lipid was found. There is no explanation for the great variability in the conversion of pyruvate to lactate in these three experiments.

Two experiments were performed in which slices were incubated with lactate-1-C^{14}, and the results are summarized in Table 9. Here it is likely that the C^{14} label was largely lost before acetyl fragments were converted to lipid and to other complex molecules. The results do indicate that the hepatoma slice can convert lactate to pyruvate as well as the normal slice can.

One of the interesting features of the pyruvate and lactate experiments is the poor gluconeogenic power of the hepatoma slice, under these experimental conditions. Such a possibility was suggested by Bierich (4) in 1928. This point is further illustrated in Chart 8, which shows that a normal liver slice from a 6-day starved rat continues to synthesize glucose, and that a net increase in glucose in the incubation flask occurs when the starved slice is incubated for 2 hours. In contrast,

TABLE 7

INCUBATION OF LIVER SLICES WITH FRUCTOSE-C^{14}

Date of exper.	Protein*			Carbon dioxide†			Lipid†		
	Normal	Hepatoma	H/N	Normal	Hepatoma	H/N	Normal	Hepatoma	H/N
2-16-51	15.9	94	5.9	1,313	1,790	1.4	298	360	1.2
3-30-51‡	19.5	160	8.2	1,700	4,200	2.5	595	814	1.4

Each flask contained 62,000 c.p.m. in 0.4 mg. fructose. Fructose prepared in this way has been found (31) to be randomly labeled. Rats were not starved. Same conditions as described in text.

* Cpm/mg protein.

† Cpm/100 mg wet wt liver.

‡ Alloxan diabetic rat, not starved. The normal represents the normal part of the hepatoma-containing liver. The rat had a blood sugar of 380 mg/100 cc.

Two experiments were performed in which slices were incubated with fructose-C^{14}. The results are summarized in Table 7. In these two experiments there appears to be less difference in the degradation to CO_2 and in the conversion to lipid between the normal liver and the hepatoma slice than was the case in the glucose-C^{14} experiments. The alloxan diabetic experiments (cf. also Table 3) failed to reveal any clear-cut distinction in the effect of diabetes on the fructose metabolism of the hepatoma slice, as compared to the normal liver slice.

Three experiments were performed in which slices were incubated with pyruvate-2-C^{14}, and the results are summarized in Table 8. Although there is considerable variation in the conditions used in these few experiments, it is clear that the extent of gluconeogenesis in the hepatoma slice is much less than in the normal slice. It also appears that there is consistently a net glycolysis when a starved (or unstarved) hepatoma slice is similarly incubated.

In carrying out multiple determinations in a complex system such as a surviving tissue slice, there are doubtless artifacts and unknown errors which influence the results. Nevertheless, it may be useful to try to fit these diverse findings into a tentative metabolic picture, as illustrated in Chart 9. In comparison with the normal hepatic slice, the hepatoma is characterized by increased glycolysis, decreased gluconeogenesis, increased conversion of pyruvate to protein, and a slight increase in oxidation of pyruvate to CO_2. There is little disturbance of the pyruvate-lactate equilibrium, if either labeled pyruvate or lactate is used as an indicator. This latter situation agrees with the conception that the increased lactic acid produc-

tion in tumors is related to an increased rate of the oxidative step of glycolysis when glucose is added.

In vivo *results.*—It is necessary to call attention to the discrepancy between our *in vitro* results and those obtained in whole animal experiments. This failure of agreement of slices and *in vivo* behavior applies both to experiments in which incorporation of C^{14}-labeled glucose and C^{14}-labeled amino acids into protein are concerned. In each instance the slice results indicate a greater rate of incorporation of the labeled compound into hepatoma protein than into control liver protein; whereas no difference is observed in the *in vivo* experiment. This situation with respect to C^{14}-labeled alanine has

TABLE 8

INCUBATION OF LIVER SLICES WITH PYRUVATE-C^{14}

	PROTEIN*			CARBON DIOXIDE*			LIPID*			LACTATE†			GLUCOSE‡		
EXPER.	Normal	Hepatoma	H/N	Normal	Hepatoma	H/N	Normal	Hepatoma	H/N	Normal	Hepatoma	H/N	Normal	Hepatoma	H/N
5-10	7.7	33	4.3	1,310	1,280	1.0				40	20	0.5	0.083	0.005	0.06
6-21	915	2,200	2.4	4,070	11,900	2.9	316	306	1.0	3.3	35	11	0.035	0.008	0.22
6-27	115	259	2.2							5.0	2.7	0.6	0.057	0.007	0.12

5-10. Rats starved 2 days, 30-minute incubation. 100,000 c.p.m. in 0.97 mg. sodium pyruvate.
6-21. Rats starved 6½ days, 2-hour incubation. 1,000,000 c.p.m. in 0.25 mg. sodium pyruvate.
6-27. Rats starved 5 days, 4-hour incubation. 100,000 c.p.m. in 0.03 mg. sodium pyruvate.

* C.p.m. per mg protein.
† γ lactate in medium after incubation/mg protein.
‡ Mg. glucose " " " " " The quantity of glucose and glycogen present in the slices was found to be a small percentage of that present in the medium.

TABLE 9

INCUBATION OF LIVER SLICES WITH LACTATE-C^{14}

	PROTEIN*			CARBON DIOXIDE*			LIPID*			PYRUVATE†			GLUCOSE‡		
EXPER.	Normal	Hepatoma	H/N	Normal	Hepatoma	H/N	Normal	Hepatoma	H/N	Normal	Hepatoma	H/N	Normal	Hepatoma	H/N
6-21-50§	648	737	1.1	16,450	10,770	0.65	145	61	0.42	2.8	3.2	1.1			
6-27-50‖	102	125	1.2							0.4	1.4	3.5	0.045	0.003	0.07

In the case of the hepatoma, it should be pointed out, there is the possibility of greater dilution of added lactate by endogenous lactate.
* Cpm/mg dry protein in flask.
† γ pyruvate in medium after incubation/mg dry protein in flask.
‡ Mg. glucose " " " " " " " "
§ Rats starved 6½ days. 0.8 mg. calcium lactate containing 1,000,000 c.p.m. per cc. medium (2 cc. in flask).
‖ Rats starved 5 days. 4-hour incubation, 0.09 mg. lactate containing 100,000 c.p.m.

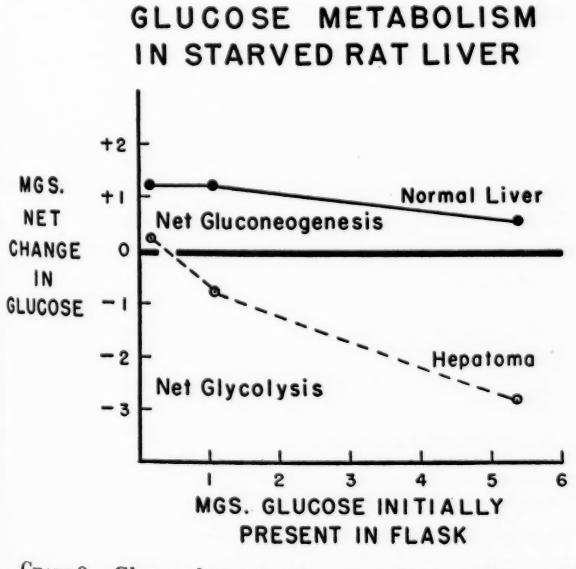


CHART 8.—Glucose determinations were done on aliquots of the media before and after incubation. The above chart represents the net change which occurred during incubation. Experimental conditions are described in text. Rats were starved 6 days.

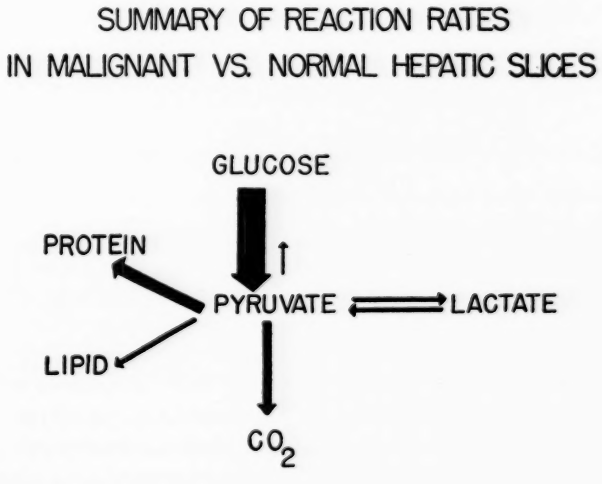


CHART 9.—The increased glycolysis, decreased gluconeogenesis, and increased conversion of pyruvate to protein in the hepatoma slice are discussed in detail in the text.

been reported (36). The data in Table 10 summarize the results of an experiment in which glucose-C^{14} and fructose-C^{14} were diluted with inert hexose and fed by stomach tube to normal and hepatoma-bearing rats. No difference in incorporation rate of C^{14} into protein was found. From the figures in the total nonprotein fraction, however, we were led to wonder whether there might be a circulatory impairment in the hepatoma.

TABLE 10

In Vivo INCORPORATION OF LABELED HEXOSE INTO RAT LIVER

	No. of rats	Protein cpm/mg dry protein	Nonprotein cpm/5 mg wet liver: Total	Nonprotein cpm/5 mg wet liver: Amino acid
Normal	3	3.7±0.3	61± 9	13±4
Control hepatoma	3	4.0±0.9	93±13	10±1
Hepatoma	3	3.7±0.4	32±11	10±3

Rats were starved 4½ days. Each rat received by stomach tube 2.5 cc. of a 48 per cent sugar solution containing an equal mixture of glucose and fructose. This contained 1.5 million counts approximately equally distributed between glucose and fructose. Rats were killed after 3 hours. The nonprotein fractions were obtained by precipitating the proteins of a 10 per cent homogenate of liver by immersing the tubes in boiling water for 7 minutes. Aliquots of the supernatant obtained after centrifugation were plated directly on planchets for counting to obtain the total nonprotein counts. Ninhydrin decarboxylation of another aliquot gave the counts present in the α-carboxyl group of the amino acids. The activity of the nonprotein fraction of plasma at the time of death was fairly constant in all the rats. The following cpm/0.1 cc plasma were obtained—hepatomas: 356, 298, and 256; controls: 270, 288, and 271.

bearing a hepatoma was injected intravenously with alanine-C^{14}, and the liver was removed and immediately frozen on CO_2 snow 1 minute later. A block of frozen tissue was cut in a modified Linderstrøm-Lang (12) freezing microtome box,[2] and sections were laid against sensitive x-ray film. These sections, plus nonradioactive control sections, were kept frozen and in the dark in contact with the x-ray film for 1 week. The radioautographs obtained (cf. Figs. 1 and 2) indicated less radioactivity in the tumor nodule than in the surrounding normal tissue. For comparison with the radioautograph, the block from which the section was cut is shown. Since it was not easy to quantitate these radioautographic findings, a type of experiment similar in general type was planned, but with certain variations to permit better quantitation of results.

A hepatoma-bearing rat was etherized, and a laparotomy was performed, exposing the liver. Alanine-C^{14} was injected intravenously into the jugular bulb, and 1 minute later the liver was removed and immediately frozen on CO_2 snow.

Neoplastic nodules were present in two lobes (Chart 10). A cylinder of frozen tissue 1 cm. in diameter and 3 cm. in length was drilled out of the large neoplasm (Chart 10, *A*) which replaced al-

TABLE 11

In Vivo INCORPORATION OF ALANINE-C^{14} INTO RAT LIVER

CONTINUOUS INFUSION OF ALANINE

Minutes	Protein* Normal	Protein* Control-hepatoma	Protein* Hepatoma	Nonprotein† Normal	Nonprotein† Total Control-hepatoma	Nonprotein† Total Hepatoma	Nonprotein† Amino acid Normal	Nonprotein† Amino acid Control hepatoma	Nonprotein† Amino acid Hepatoma
2	0.43	0.30	0.27	110	38.4	26.1	72	37	24
6	1.39	0.75	0.25	211			113		22
10	2.91	1.81	0.80	376	306	209	246	172	117

The normal rat (190 gm.) received by jugular vein 1 cc. of a 1 M solution of alanine-C^{14} in saline containing 4,160,000 c.p.m., at the rate of 0.1 cc. per minute. The hepatoma (234 gm.) received 1.2 cc. of the same solution at the rate of 0.12 cc. per minute. Thus each rat received the same amount of solution per gram of body weight. Aliquots of hepatic tissue were removed at 2, 6, and 10 minutes and were immediately frozen on CO_2 snow. Blood was withdrawn from the heart of each animal immediately after the flow of alanine was stopped (at 10 minutes). The following nonprotein c.p.m. per 0.1 cc. plasma were obtained: normal, 11,000 c.p.m.; hepatoma, 8,700 c.p.m. The tissues were treated as in Table 10.

* Cpm/mg dry protein.

† Cpm/5 mg wet liver.

An experiment was therefore carried out in which alanine-C^{14} was used as a convenient measure of circulation to the hepatoma and normal liver. A continuous infusion of a solution of alanine-C^{14} was given intravenously over a 10-minute period into the jugular vein of an etherized rat bearing a hepatoma, and similarly into a normal control rat. Aliquots of hepatic tissues were removed at 2 and 6 minutes, and the animals were killed at 10 minutes. The results shown in Table 11 suggest that the circulation to the hepatoma is impaired.

As an independent test of this question, a rat

most one entire lobe. Frozen sections 25 μ thick were cut from this cylinder in the freezing box, and a "radioactivity histogram" was constructed, after counting the dried sections on copper planchets.

As a repeating pattern three sections were mounted on discs for counting, two were mounted on slides for staining, and the equivalent of 60 sections was saved for fractionation into protein and nonprotein fractions, all the way through the

[2] Mechanical refrigeration was substituted for dry ice cooling.

nodule. The histological sections revealed extensive necrosis in the center of the nodule, but very little necrosis in the periphery.

A similar plug of tissue 1 cm. in diameter was cut from a nonmalignant lobe (*B*) of the liver. The most interesting finding was that *the specific radioactivity per milligram of tissue was greater in the normal lobe* (B) *than in the periphery of the neoplastic tissue*, an average of 1,100 c.p.m. per section in the nonmalignant slice, as compared to 600 c.p.m. in the malignant slice near the periphery of the nodule. The results (cf. Chart 11) also indicated that the circulation of this large tumor nodule was confined to the periphery and that the

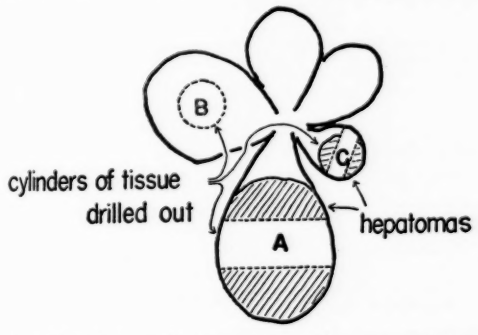


CHART 10.—Schematic drawing of liver containing hepatoma nodules. The shaded areas represent hepatoma nodules. Plugs of tissue removed from areas A, B, and C, are described in the text.

center was avascular. Thus, the histogram provides a biochemical description of an important factor in the production of necrosis.

A plug of tissue 0.5 cm. in diameter was cut through a smaller hepatoma nodule (*C*) and adjacent tissue. Slices cut through the smaller nodule revealed, in contrast to the situation in the larger nodule, however, that essentially the same specific radioactivity was present throughout the nodule, and the histological sections showed little necrosis, even in the center of the nodule. Thus, in these two tumor nodules it would appear that the vascular supply comes from the periphery and is able to penetrate to a depth of 5 mm., but beyond there the vital activity of the neoplastic tissue becomes imperiled by circulatory inadequacy.

These *in vivo* experiments suggest that the neoplastic hepatic tissue operates under restrictions imposed by a circulatory impairment. Since the blood brings oxygen, amino acids, and a carbohydrate energy source, all of which are needed, according to present views, for protein synthesis, it would appear that a small decrease in blood flow to a tissue might result in a larger percentage decrease in protein synthesis. It is possible, although still conjectural, that the slice experiments compare the *capacities* of the normal and neoplastic tissues to incorporate amino acids and convert glucose into protein; whereas the whole animal experiments compare these capacities as they exist under the *restrictions* imposed by the circulation.

SUMMARY

These experiments fail to disclose any qualitative differences in metabolic pathways followed by the neoplastic and normal hepatic tissues. They

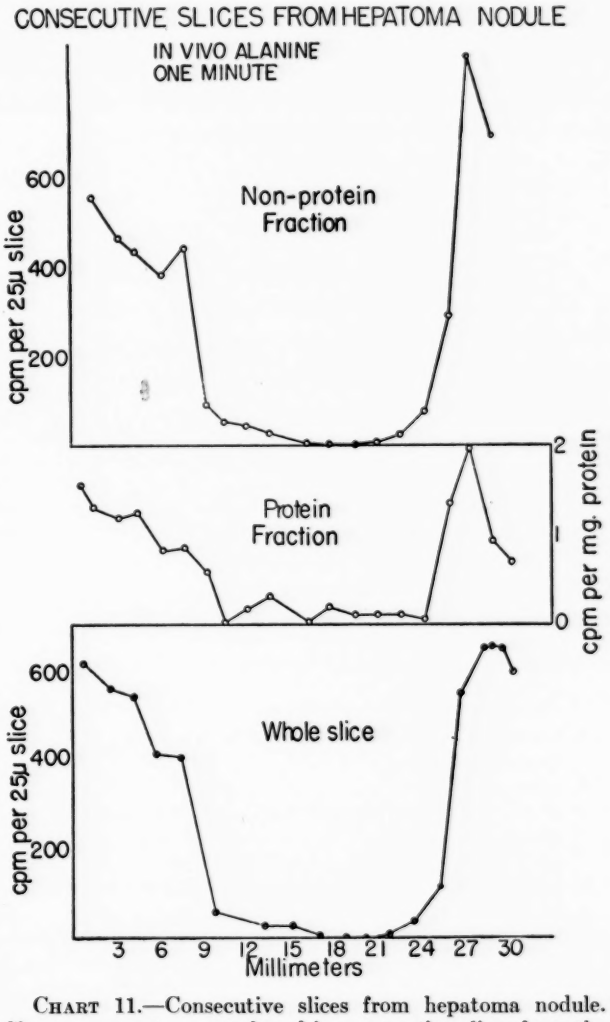


CHART 11.—Consecutive slices from hepatoma nodule. Chart represents counts found in consecutive slices from the large hepatoma nodule (*A*) shown in Chart 10. Rat was given 0.45 cc. alanine-C^{14} containing 50,000,000 c.p.m. in 50 mg. by jugular vein. One minute later the large hepatoma nodule and a normal lobe were removed and frozen on CO_2 snow. A cylinder of frozen tissue going from one edge of the nodule to the other was cut out of the hepatoma. A No. 8 cork borer was used. This gave a diameter of about 1 cm. The "whole slice" counts were obtained by placing a frozen 25 μ slice on a copper planchet. The slice was dried at room temperature and counted. The pooled 60-slice fractions were homogenized in 3 cc. of H_2O, and the tubes were immersed in boiling water for 7 minutes to precipitate the protein. The supernatant obtained after contrifugation was plated on planchets. This represented the nonprotein fraction. The protein was washed the usual way and plated on discs for counting.

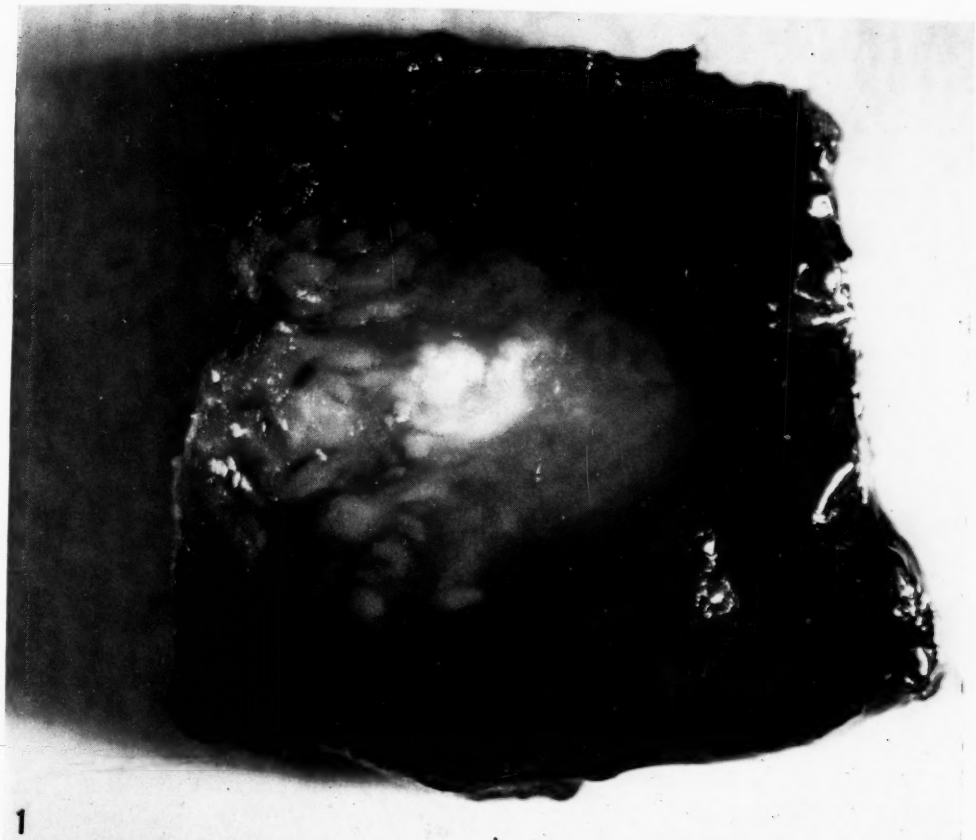
do appear to imply that the synthetic activities of the hepatoma slice are adjusted more toward construction of molecules such as proteins which are immediately concerned with cell growth than toward storage of energy-rich material such as glycogen for future use. The previous inability to correlate *in vitro* and *in vivo* experiments on amino acid incorporation into protein has been related to a circulatory impairment of the tumor in the living animal.

ACKNOWLEDGMENTS

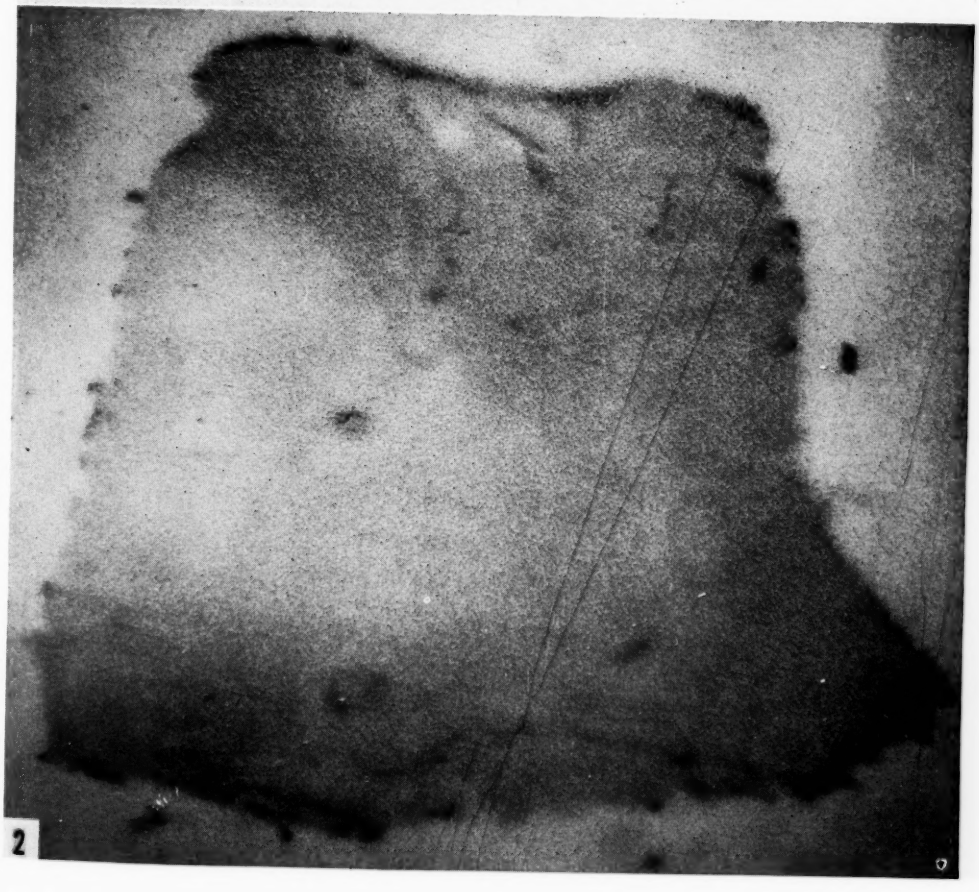
The authors wish to thank Dr. Joseph C. Aub for encouragement and fine laboratory facilities, and Dr. Elizabeth Keller for helpful advice and criticism.

REFERENCES

1. Anfinsen, C. B.; Beloff, A.; Hastings, A. B.; and Solomon, A. K. The *In Vitro* Turnover of Dicarboxylic Amino Acids in Liver Slice Proteins. J. Biol. Chem., **168**: 771–72, 1947.
2. Anker, H. S. Synthesis of Carbonyl-labeled Pyruvic Acid. J. Biol. Chem., **176**:1333–35, 1948.
3. Barker, S. B., and Summerson, W. H. The Colorimetric Determination of Lactic Acid in Biological Material. J. Biol. Chem., **138**:535–54, 1941.
4. Bierich, R. The Lactic Acid Enrichment in Malignant Tumors, p. 61. Internat. Conf. Cancer, London, 1928.
5. Calvin, M., and Lemmon, R. M. Decarbonylation of Ethyl Pyruvate. J. Am. Chem. Soc., **69**:1232–33, 1947.
6. Cramer, R. D., and Kistiakowsky, G. B. Synthesis of Radioactive Lactic Acid. J. Biol. Chem., **137**:549–53, 1941.
7. Frantz, I. D., Jr., and Zamecnik, P. C. Use of C^{14}-labeled Amino Acids in the Study of Peptide Bond Synthesis. In: Symposium on Plasma Proteins, pp. 94–107. Springfield: Charles C. Thomas, 1950.
8. Friedemann, T. E., and Haugen, G. E. Pyruvic Acid; Determination of Keto Acids in Blood and Urine. J. Biol. Chem., **147**:415–42, 1943.
9. Good, C. A.; Kramer, H.; and Somogyi, M. Determination of Glycogen. J. Biol. Chem., **100**:485–91, 1933.
10. Henriques, F. C., Jr.; Kistiakowsky, G. B.; Margnetti, C.; and Schneider, W. G. Radioactive Studies. I. Analytical Procedure for Measurement of Long-lived Radioactive Sulfur, S^{35}, with a Lauritzen Electroscope and Comparison of Electroscope with Special Geiger Counter. Ind. Eng. Chem., Anal. Ed., **18**:349–53, 1946.
11. Koshland, D. E., Jr., and Westheimer, F. H. Mechanism of Alcoholic Fermentation. The Fermentation of Glucose-1-C^{14}. J. Am. Chem. Soc., **72**:3383–88, 1950.
12. Linderstrøm-Lang, K., and Mogensen, K. R. Studies on Enzymatic Histochemistry. XXXI. Histological Control of Histochemical Investigations. Comptes Rendus des Travaux du Laboratoire Carlsberg, **23**:27–35, 1938.
13. Loftfield, R. B. Preparation of C^{14}-labeled Hydrogen Cyanide, Alanine, and Glycine. Nucleonics, **1**:54–57,1947.
14. Magasanik, B., and Umbarger, H. E. Separation and Identification of Ketoacids by Filter Paper Chromatography. J. Am. Chem. Soc., **72**:2308, 1950.
15. Mahler, H. R. Synthesis of D-Glucose-1-C^{14}. A. E. C. Document 2400, 1948.
16. Melchior, J. B., and Tarver, H. Studies in Protein Synthesis *in Vitro*. I. On the Synthesis of Labeled Cystine (S^{35}) and Its Attempted Use as a Tool in the Study of Protein Synthesis. Arch. Biochem., **12**:301–8, 1947.
17. Moore, S., and Stein, W. H. Photometric Ninhydrin Method for Use in the Chromatography of Amino Acids. J. Biol. Chem., **176**:367–88, 1948.
18. ———. Chromatography of Amino Acids on Starch Columns. Solvent Mixtures for the Fractionation of Protein Hydrolysates. *Ibid.*, **178**:53–77, 1949.
19. Nelson, N. A Photometric Adaptation of the Somogyi Method for the Determination of Glucose. J. Biol. Chem., **153**:375–80, 1944.
20. Noggle, G. R., and Bolomey, R. A. The Biosynthesis of C^{14}-labeled Compounds. I. The Chromatographic Separation of Glucose and Fructose. Plant Physiol., **26**:174–81, 1951.
21. Partridge, S. M. Application of the Paper Partition Chromatogram to the Qualitative Analysis of Reducing Sugars. Nature, **158**:270, 1946.
22. ———. Filter-Paper Partition Chromatography of Sugars. Biochem. J., **42**:238–48, 1948.
23. ———. Aniline Hydrogen Phthalate as a Spraying Reagent for Chromatography of Sugars. Nature, **164**:443–44, 1949.
24. Pucher, G. W.; Leavenworth, C. S.; and Vickery, H. B. Determination of Starch in Plant Tissues. Ind. Eng. Chem., Anal. Ed., **20**:850–53, 1948.
25. Putnam, E. W.; Hassid, W. Z.; Krotkov, G.; and Barker, H. A. Preparation of Radioactive Carbon-labeled Sugars by Photosynthesis. J. Biol. Chem., **173**:785–95, 1948.
26. Roe, J. H. A Colorimetric Method for the Determination of Fructose in Blood and Urine. J. Biol. Chem., **107**:15–22, 1934.
27. Siekevitz, P. Thesis, University of California, Berkeley, 1949.
28. Sowden, J. C. Condensation of C^{14}-Nitromethane with D-Arabinose: Preparation of 1-C^{14}-D-Glucose and 1-C^{14}-D-Mannose. J. Biol. Chem., **180**:55–58, 1949.
29. Stein, W. H., and Moore, S. Chromatography of Amino Acids on Starch Columns. Separation of Phenylalanine, Leucine, Isoleucine, Methionine, Tyrosine, and Valine. J. Biol. Chem., **176**:337–65, 1948.
30. Tschelinzeff, W., and Schmidt, W. Über ein Neues Verfahren zur Darstellung von α-Ketonsäuren. Ber., **62**:2210–11, 1929.
31. Udenfriend, S., and Gibbs, M. Preparation of C^{14} Uniformly Labeled Fructose by Means of Photosynthesis and Paper Chromatography. Science, **110**:708–9, 1949.
32. Umbreit, W. W.; Burris, R. H.; and Stauffer, J. F. Manometric Techniques and Related Methods for the Study of Tissue Metabolism, p. 192. Minneapolis: Burgess Publishing Co., 1949.
33. Villee, C. A., and Hastings, A. B. The Metabolism of C^{14}-labeled Glucose by the Rat Diaphragm *in Vitro*. J. Biol. Chem., **179**:673–87, 1949.
34. Warburg, O. The Metabolism of Tumours, translated by F. Dickens. New York: R. R. Smith, Inc., 1931.
35. Winzler, R. J.; Krause, R.; and Slater, G. G. Aerobic and Anaerobic Incorporation of Radioactive Phosphate and Radioactive Glucose Fragments by Normal and Cancer Tissue *in Vitro*. Cancer Research, **10**:249, 1950.
36. Zamecnik, P. C., and Frantz, I. D., Jr. Peptide Bond Synthesis in Normal and Malignant Tissue. Cold Spring Harbor Symp. Quant. Biol., **14**:199–208, 1949.
37. Zamecnik, P. C.; Frantz, I. D., Jr.; Loftfield, R. B.; and Stephenson, M. L. Incorporation *in Vitro* of Radioactive Carbon from Carboxyl-labeled DL-Alanine and Glycine into Proteins of Normal and Malignant Rat Liver. J. Biol. Chem., **175**:299–314, 1948.
38. Zamecnik, P. C., and Stephenson, M. L. Studies on Cross-Connections between Carbohydrate and Protein Metabolism in the Rat Hepatoma. Cancer Research, **10**: 251, 1950.



1



2

FIG. 1.—Photograph of rat liver tissue block containing a hepatoma. This is shown, rather than the actual section used for the radioautograph, for greater ease in identifying the tumor nodule.

FIG. 2.—Radioautograph of rat liver containing a hepatoma.

FIGS. 1 AND 2.—The liver of an etherized rat bearing a hepatoma was exposed; 0.5 cc. of a solution of alanine containing 40,000,000 c.p.m. in 40 mg. was injected into the tail vein. One minute later, the liver was removed and immediately frozen on CO_2 snow. A block of tissue containing a hepatoma nodule plus some normal tissue was used.

Trends in Cancer Research*

E. V. Cowdry

(Department of Anatomy and Wernse Cancer Research Laboratory, Washington University, St. Louis, Mo.)

Most presidents of this Association have on these occasions considered an aspect of cancer research in which they themselves have made notable advances. These presentations have all been valuable. In many cases announcements of important discoveries have then been made for the first time. These have been immediately communicated to the public by an alert press to which we extend our compliments and our thanks.

The members of the Association, and their guests, have felt privileged to listen. Yet some of them could understand but imperfectly. Progress in cancer research is made by specialists in many lines of endeavor. These specialists are immersed in their tasks. They use a language all their own, and they have a tendency to credit workers in other lines with more knowledge than they actually possess. Consequently these presidential addresses are for some of us just grand and for others on too high a plane—obviously, we can't all be equally versed in genetics, chemistry, physics, cytology, and clinical medicine, to mention only a few of them. No one brain can encompass the cancer field. An Aristotle would be floored.

I could try to follow suit and speak to you about the studies on epidermal carcinogenesis carried on by our small group since 1938; but something tells me that most of you would be bored. Instead, I shall try to follow the example of my respected predecessor, Dr. Aub. You will remember his eloquent address, "This Business of Cancer Research." It was a huge success and appealed to all of us. Being constituted as I am, I am unable to supply anything approaching his sparkling humor. I am advised, wisely, that anything of that kind from me would fall flat.

"Trends in Cancer Research" is a safe topic. The only danger is in frankness, and this I do not intend to avoid. My approach has been to induce the members of this Association to speak up for themselves and to bring before you their opinions. A questionnaire was sent out, with the announcement of this meeting, by our secretary, Dr. Hooker, who incidentally contributes more than anyone else to our Association. The thanks of all of us are due to him.

To begin with we asked our members to identify their line of principal research activity in the cancer field—genetics, biochemistry, chemotherapy, pathology, endocrinology, radiology, nutrition, or whatever it happens to be. The thought was that this would indicate their points of view of the cancer problem. Most of them stated that they were active in several fields.

Then we requested their opinion on specific questions. The report of a committee on "Fundamental Cancer Research" to the Surgeon General in 1938 constitutes a landmark, so this was taken as a good point of departure. The questions were:

1. *What are the chief achievements since 1938 in your field?*

As is to be expected, the answers covered a wide range of activity, and there was little consensus of opinion. They included:

The development of cancer in tissue cultures.
The introduction of single cell cultures.
The transplantation of human cancer into other species.
Hormone treatment of prostatic and breast cancer.
The identification of the milk factor as a virus.
Demonstration of transmissible cytoplasmic characters.
Knowledge of submicroscopic structure revealed by electron microscopy.
Advances in the study of metabolic antagonists.
Use of radioactive carcinogens.
Progress in the early diagnosis of human cancer by cell smears.
Demonstration of association between excessive cigarette smoking and lung cancer.
Large scale testing of possible chemotherapeutic compounds.
Proof that dietary factors influence cancer production in animals.
Elucidation of the function of mitochondria, especially in the concentration in them of certain enzymes.

* Presidential address delivered at the Forty-second Annual Meeting of the American Association for Cancer Research, Inc., Cleveland, Ohio, April 28, 1951.

Demonstration of the effect of specific genes on the control of susceptibility to tumor formation.

Demonstration of the association of specific genes with antigens controlling the transplantation of tumors.

Demonstration of the influence of hereditary factors in cancer production in animals.

The discovery that genetic changes result from the action of chemical carcinogens as well as from radiation.

The bringing of the chemotherapeutic agent more directly to bear on the cancer in human beings by intra-arterial injection.

Recognition of a qualitative difference between malignant and normal tissue.

From these and other answers one might easily get the impression that we have made great strides forward since 1938, that the goal is just around the corner. But sober thought tempers this complacent feeling of satisfaction. There have been advances. It can be said that we are coming to grips with the cancer problem. We are, however, a long, long way from solving it.

2. *What are the present trends?*

Chemotherapy was placed first by a wide margin. Also mentioned were: endocrine balance, submicroscopic structure, antimetabolites, viruses, host-tumor relationships, and numerous other fields of endeavor. Evidently in the past 12 years it has not been discovered where the major offensive against cancer should be staged. If agreement could have been reached on the strategy likely to achieve success, generous financial support would have been forthcoming.

3. *What others seem most worthy of exploration?*

Many of the members naturally thought that their own work should be pushed, but some gave other lines of investigation including:

The mechanism of metastasis.

Blood diagnostic tests.

Inheritance of cancer in man.

Clinical experimentation on advanced cases of human cancer.

4. *Are any trends in your field being supported and over-emphasized at the expense of others apparently more fruitful?*

Most of the members avoided this question, some pleading ignorance, but the following opinions were recorded:

Too many programs. The idea is that these programs are designed to catch the dollars and are in a sense artificial.

Gross chemical and enzyme compositions of normal versus tumor tissue.

Production of tumors in animals by powerful synthetic carcinogens. There is little use in merely adding to the already long list unless the new carcinogen behaves in an especially revealing fashion. These powerful synthetic carcinogens are not the ones that cause cancer in man.

Heat coagulation of blood serum as a diagnostic test.

Animal genetics.

Many of the answers to the questionnaire were carefully prepared, and I am grateful for them. The task of writing all the letters of thanks has thus far been too great for me.

On the whole, the number of answers was disappointing. I do not think that this indicates indifference on the part of our members. None of the members of the Association belonging to Washington University answered. I mentioned this to a friend who said: "Oh, they know that you know their opinions anyway; they will be glad to answer if you want them to; shall I ask them to?"

But I do interpret this rather general failure to respond as an expression of a widespread feeling of futility. Our members have opinions, but most of them don't think that anything is to be gained by coming out with their views. The American Association for Cancer Research is, after all, the largest and most active national organization in the world devoted to cancer research. It is, in my humble opinion, an unhealthy condition for this Association to remain passive while large and powerful organizations devoted to financing cancer research decide which investigations are to be promoted by financial contributions and which are to be starved to death by lack of funds. Self-analysis and self-criticism can be helpful. However, the situation is not as serious as it might seem to be at first sight, for these organizations depend to a large extent on our members as consultants. The opinion of the Association is voiced through them. The American Association for Cancer Research does not realize its power.

Speaking now frankly, as an individual, I have certain opinions for which I take the responsibility. It seems to me that the report already alluded to by Bayne-Jones, Harrison, Little, Northrop, and Murphy to the Surgeon General in 1938 is equally valid today. As I have already admitted, the progress made since that time has been disappointing. We still await the discovery of weakness on the part of cancer cells, or of chinks in their armor through which they can be attacked. Leukemias and cancer that have spread throughout the body are as deadly today as they were in

1938. We can delay the outcome and reduce suffering to some extent, but beyond that we remain powerless. Despite many promising experiments, chiefly with animals, and promptly publicized, we repeatedly are forced to admit that the fates of thousands remain sealed.

But our approaches to the cancer problem change. We can recognize the appearance since 1938 of some definite trends of interest and of emphasis which did not come out in the answers because the questions were not adequately phrased.

The pioneer foundations are to be congratulated for their initial leadership. I have in mind the Donner Foundation, The Jane Coffin Childs Memorial Fund for Medical Research, The Anna Fuller Fund, and several others. When, however, powerful impetus was given to cancer research, first by the National Cancer Institute, and later by the rejuvenated American Cancer Society, it was perfectly clear that we were trying to advance on too narrow a front. Sciences that could bring to bear new technics and points of view on the cancer problem were not being explored, and some leaders in them—immersed in their own affairs—remained unaware of their ability to help.

It was logical to expand this front by directing attention to cancer and by encouraging investigators in many lines of research to concentrate on it. Some of these workers were surprised and pleased to learn how important in the cause of cancer their studies were regarded. It was not infrequently explained to them that, after all, no change in their researches was wanted, because they were already obtaining potentially significant data on fundamentals such as enzyme activity, the mechanism of cell division, and so on. All that was required of them was to accept more money and to work more diligently. The expansive trend was not only to utilize a wider range of technics and specialists but also to activate institutions throughout the United States, both strong and weak, willing to co-operate—in brief, it was to give opportunities to many.

This wise policy has paid dividends. Now the trend is to decrease the spread of funds far and near in order to fertilize cancer research in the sciences, and to concentrate on projects which appear to be most important and feasible, as, for example, chemotherapy and the differences between normal and malignant cells which may be expected to afford clues as to the specific vulnerability of cancer cells.

From the beginning, clinicians have been aware of their responsibilities. They have not been idle, but for years they have been handicapped by lack of financial support because principal emphasis has been given to so-called fundamental research on animals in which controlled experiments are more feasible. Some have complained bitterly to me. They agree that this fundamental research must not be relaxed. However they enthusiastically welcome the trend to encourage clinical research designed to secure more prompt diagnosis and to utilize without delay all possible means to help patients with advanced cancer. This overdue shift of emphasis to include clinical research is an important sign of the times.

In addition to this increase in clinical research, we note, both in the clinic and the laboratory, an inclination to take more seriously the concept of the virus etiology of cancer. This trend results from several discoveries: *first*, the proof that more and more cancers in animals are caused by viruses; *second*, the clear demonstration that a virus may produce tumors in adults and an acute disease not apparently neoplastic in the young; and *third*, the visualization of an increasing number of viruses by electron microscopy. There is also the fact that the milk factor, now identified as a virus, would never have been discovered had it not been possible in mice to establish highly inbred strains. Viruses in human cancer may remain hidden because this method of identification is not possible. Hereditary factors in human cancer may also remain hidden for the same reason. In my opinion the virus theory, if substantiated as the cause of some cancers, will open the way to prevention and treatment. But I do not consider it likely that all cancers are caused by viruses.

Still another trend in cancer research is an encouraging increase in international co-operation. You will agree that there is no racial or geographic monopoly of wisdom. All can, and should, take part in this attack on a common adversary. At the Cancer Congress in 1947 in St. Louis, the International Cancer Research Commission was born. This is a landmark of consequence equal to the Report of 1938 to the Surgeon General to which I have frequently alluded. The Commission is the research arm of the International Union Against Cancer. At first it was very coldly received in this country and enthusiastically abroad. We, here, have a special responsibility in its support, because two world wars have left us the most wealthy of nations. I am happy to say that opinion is definitely veering in the United States toward the promotion of international teamwork in cancer research to the advantage of humanity everywhere. Those, who say rather truculently: "We have both the brains and the equipment, why waste time and money on outsiders?" are short-sighted isolationists.

Now let us ask ourselves some additional ques-

tions in the firm belief that our ways of doing things, in many respects excellent, can be improved.

1. *What do most of us consider the principal handicap under which we are working?*

The answer will be, I am sure: "Worry about how we can carry on from year to year." There has been some improvement, because an increasing number of grants are made for more than 1 year. Though a guarantee of continuation is often lacking, when the time comes there is a continuation; but until then the insecurity is nerve-racking. I speak feelingly, because this has happened to me, despite the fact that the National Cancer Institute continued to support my work I think longer than that of any other individual. Young investigators, fired by enthusiasm, devote themselves to a career of full time cancer research at their peril. If they stay in it a long time, and many years slip by, they may wake up to a realization of the fact that they are no longer in line for important university appointments.

The trend is unhappily not toward the endowment of cancer research. When the Rockefeller Foundation became convinced that some special division of medicine should be developed it did not follow the results-quick technic of annual grants. It provided endowment. Unhappily the interest yield on endowment has steadily decreased, yet absence of endowment for cancer research is still the principal handicap.

2. *Has it become easier to finance cancer research?*

If by this is meant temporary financing, the answer will depend upon the time the supplicant has been engaged in cancer research. If he is doing satisfactory work in some science that can conceivably contribute significantly to cancer, he has a good chance to get a start. The trend to expansion of the research front, though reduced, still operates. If he has been doing good work in cancer research for 10 years or more, it is not so easy. It is said that his university should support him, that others should be given a chance. On the whole, those whose task it is to direct cancer research laboratories are finding this task increasingly difficult. They will concur that this is a trend in the wrong direction.

3. *Has the mechanism of obtaining grants for cancer research been simplified and the delay reduced?*

The first answer is that it has become more complicated. I am sorry that I can't show you tonight samples of the application forms used in 1938 and in 1951. The first were a few sheets, the latter constitute small volumes. Stacked up they would reach to the ceiling. The first were appraised by a small group of experts, the latter by a series of panels, or study units, including hundreds of persons. To begin with, an answer could be secured promptly by writing or telephoning to those best able to judge.

Now, with increasing complexity, the delay is frustrating. Moreover, the problem of making progress reports has not been simplified. The financing organizations ask for complete *in extenso* progress reports and a great many copies. But one wise foundation requests a report of one page, or at most two pages, and only one copy. You will agree that this is a move calculated to ease the burden on us, the investigators. It also serves to improve the quality of the report. It means that the investigators weigh very carefully every word and that the job of the recipients of the reports is simplified.

For this reason, I advocate this single sheet method of making progress reports and applications for funds. Objections will assuredly be raised by those who hate simplification and streamlining. If forced to, we might meet them half-way and urge the use of two forms: (*a*) a long form to be used for the first application by beginners unknown to the judges concerning whom detailed information is needed and (*b*) a short, single-page form for subsequent applications and reports by these beginners and for all applications and reports by experienced workers already well known to the judges.

I suggest, in the second place, that financing organizations reduce the number of experts in study units and panels by about 50 per cent. Over 100 advisors on any medical problem are too many. They get in each other's way. The present method is democratic, for it gives a large number of experts, regularly being replaced, whose opinions are valuable, a chance to express their views. But the said experts are busy people working for the love of it in the little time that they can spare. We are greatly indebted to them. It is not surprising that executive officers hesitate to bother them between meetings.

However, efficiency would be promoted without great sacrifice of democratic procedure if a few of these outstanding leaders were invited to devote a year to the job full time on salary, as was the original policy of the National Research Council. Some would accept and these could take the place of many. Please understand me. I am not advocating abandonment of an excellent system. Numerous advisors should be retained, working in their spare time, strengthened by others serving temporarily full time.

It would then be possible for the financing organizations very promptly to advise applicants

informally whether their requests for aid are likely to receive serious consideration. Time is precious. To be obliged to wait for several months expectantly for the verdict when, for some reason unknown to the applicant, it is clear to those in authority that the answer will be unfavorable is a disservice to cancer research. The application should not be declined, but the applicant should be informally advised to look elsewhere. One foundation, that I know well, operates on this basis to the satisfaction of all concerned.

I realize that I only have a short time longer during which I am privileged to speak as your President. In stating my views frankly I am talking to you as members of the American Association for Cancer Research who must make applications for grants. I am also addressing you as members of panels and study units who receive applications and make recommendations. It seems to me that serving in the latter capacity there are two duties: *first* to recommend the disbursement of funds wisely as I know that you do, and *second* to advise the adoption of changes which will make the task of yourselves and of your friends as applicants and investigators easier.

All of us probably can think of other means of simplification in addition to those already mentioned. Progress reports should not only be abbreviated, they should also be reduced to one per annum. Since it is the custom of organizations receiving applications to require information about all applications to other organizations, which they freely exchange among themselves, a single annual progress report should include all advances made by the particular investigator or group each year, irrespective of the sources of the financial aid but of course mentioning them. At one stroke this would give them a complete and integrated account of progress. It would also save the hard-pressed worker from making individual reports to each of them.

There is still another way in which the energy of the cancer investigator can be conserved so that it can better be concentrated on his work. It is by arrangement between the financing bodies to agree on the same fiscal year. At present my books have to be kept on years beginning January 1, July 1, and September 1. Please advise the elimination of this handicap.

I repeat, this Association does not realize its power to help itself and in so doing to advance cancer research.

Having in mind our primary objectives of preventing and curing cancer, a closer approach to the task is indicated. At present, financing organizations receive hundreds of applications for financial aid, and they consider them long, meticulously, and wisely. In a word, they are passive recipients. In addition, a more direct method to reach the goal is to follow the example afforded by the Office of Scientific Research and Development (OSRD) in World War II. This office decided what was wanted, selected the individuals who could help most, and urged them and their institutions to join the team. The trend I would like to see is to supplement the cultivation of individual initiative, which means so much to all of us, by promoting an organized and sustained attack on cancer. Some disbursing organizations already have directives actively to invite people to undertake investigations considered important. Perhaps they even have carefully matured plans of just how, in their judgment, cancer should be attacked. Action is urgent. A five-, or a five-and-a-half-day week is not sufficient. To lose time unnecessarily is almost criminal; cancer victims go on dying.

Studies on Lymphocystis Tumor Cells of Fish

II. Granular Structures of the Inclusion Substance as Stages of the Developmental Cycle of the Lymphocystis Virus

RICHARD WEISSENBERG

(Department of Anatomy, Woman's Medical College of Pennsylvania, Philadelphia 29, Pa.)

The lymphocystis tumors of fish consist of round, uninucleate cells of gigantic size which in the perch Stizostedion can reach 1,200 μ in diameter, and in the flounder Pleuronectes almost 2,000 μ. They are not parasitic protozoa as Woodcock supposed, who described them in 1904 as *Lymphocystis johnstonei* Woodc. (15), but are actually fibroblasts of the fish which have undergone an enormous hypertrophy (9, 10, 11). The cytoplasm of these "lymphocystis cells" contains basophilic inclusion bodies which can reach very large dimensions but start their development as tiny corpuscles. This observation, together with the fact that the lymphocystis disease proved to be very infectious, clearly indicated that the disease is produced by a virus which is located in the hypertrophying cells.

Recently specific granules were demonstrated in the outer layers of the inclusion bodies (14). The granules are stained dark brown when exposed to an osmic acid solution for several hours or longer. Because these components of the inclusions were small elements of rather uniform size, which increase considerably in number during the growth of the lymphocystis cells, it was concluded that they probably represented the transmission stage of the virus, to be compared with the elementary bodies of macroviruses (e.g., the viruses of vaccinia, fowl-pox, psittacosis), which are likewise visible under the light microscope. Any suspicion that the "osmiophilic granules" might be artifacts, produced by osmium precipitations, was dispelled by their demonstration as refractive structures in areas unstained by the osmium, and further by the fact that similar granules were seen in fresh preparations when the cytoplasm was made transparent by adding drops of dilute acetic acid.

Because the osmiophilic granules could not be demonstrated in the early period of the development of the inclusion bodies, the possibility was suggested that they were formed at intermediate stages, as differentiation products of the inclusion substance. However, details of such a process were not known. It was a problem whether the granules appear abruptly or whether certain microstructures in the inclusion substance are precursors to initiate the process. The present paper attempts to answer these questions and thus to contribute to the knowledge of the developmental cycle of the lymphocystis virus by a study of finer structures of the inclusion substance in stages preceding the appearance of the osmiophilic granules, and by a detailed investigation of the following stage in which osmiophilic granules have just made their appearance.

Received for publication September 20, 1950.

MATERIALS AND METHODS

For this morphological study lymphocystis tumor material was used from three fish species, the American perch *Stizostedion vitreum* and two European fish from the Baltic Sea: the perch *Acerina cernua* and the flounder *Pleuronectes flesus*. The material consists partly of collected fish specimens bearing spontaneous lymphocystis tumors, and partly of fish with experimental infection. Various methods of fixation and staining were applied (see legends of figures). The methods of staining with osmium have been described in detail (14).

RESULTS

Figures 1–5 show successive stages of the growth of inclusion bodies studied in young Pleuronectes lymphocystis cells of 40–200 μ in diameter which were obtained by experimental infection. In addition to the increase in size, the appearance of vacuoles and the formation of large inner spaces can be observed. When the superficial layer ruptures, as

shown in Figure 2 at the points marked by arrows, indentations are formed which may be followed later by fenestrations when the inclusion body further expands into the cytoplasm. The basophilic inclusion substance, in which the vacuoles are imbedded, could not be resolved into finer structures in the preparations shown in Figures 1 and 2, which are stained with hematoxylin after fixation in acetified alcohol. In the inclusion (Fig. 3) which was fixed in Meves' fluid, a number of highly refractive granules can be seen stained intensely with safranin. They are loosely scattered among the vacuoles. Their number is variable, and in many inclusions they are not present at all. In several inclusion bodies of the same material, concrements with a diameter of up to 1.5 μ were observed which likewise stain with safranin and have a high index of refraction. Under these circumstances, it seems to be quite possible that these granules have only the significance of small concrements.

The Sjövall method, by which in more advanced stages the osmiophilic granules can be demonstrated, makes the inclusion substance often appear as a rather homogeneous mass lightly stained by the osmium (Fig. 4). However, a continued study of such Sjövall preparations has shown that, in addition to the "clear type" of inclusion bodies, another type also occurs in which the inclusion substance shows a brown color as the result of the osmium staining. In thin layers of such "brown inclusions" a mosaic of dull brown granules could be discerned by use of oil immersion lenses of high aperture, as shown in (*a*), (*b*), (*c*), and (*d*) of Figure 5. The granules are imbedded in a lightly stained, delicate intergranular substance. These granular components of the inclusion substance are, in comparison with the osmiophilic granules, characterized by a less intensive staining reaction with osmic acid and a lower index of refraction.

Tumors of flounders caught in the fall showed the lymphocystis cells in the next period of their growth in which they reach 220–270 μ in diameter. In the tumors of one freshly fixed specimen a number of degenerated lymphocystis cells was seen. Furthermore, in many cells the inclusions could be noted in a peculiar process of swelling which gave them the appearance of fluffy bodies. Early stages of the swelling are seen in Figure 6, advanced stages in Figure 9. With such simple methods as fixation in alcohol and staining with hematoxylin, a granular composition of the inclusion substance could distinctly be seen in the fluffy bodies, even with an ordinary oil immersion lens in combination with a low power ocular. The inclusion body (*a*) of Figure 6 is shown in Figure 8 in high magnification. Between vacuoles a large number of intensely stained granules is seen, often arranged in tetrads (for instance, at the arrow) or dyads and short chains. In the group of very small inclusion bodies marked (*b*) in Figure 6, which is shown in Figure 7 in high magnification, again such a pattern of intensely stained granules can be seen imbedded in a delicate intergranular substance. The inclusion body (b^1) shows one tetrad in the central area. In (b^2) several tetrads and dyads are seen. Some of the granules appear somewhat elongated, as short rods. In advanced stages of the swelling, as shown in Figure 9 at (*f*), the granules appear in high magnification (Fig. 10) as more lightly stained structures. Again tetrads and dyads with granules in a tandem position are frequently observed. In an interpretation and evaluation of patterns of granules there is, of course, always some danger of overemphasizing a topographic relation of granules which may only by chance lie close together. The arrangement of the granules can be noted in the characteristic groups of tetrads so often, however, that one cannot consider such arrangements as merely incidental. Thus, the presented evidence indicates that the granules are units which multiply by fission.

Secondary application of osmic acid solution to the alcohol-fixed material of the swollen inclusion bodies resulted in an osmium staining of the granules which, in its rather low intensity, contrasted to the characteristic staining reaction of osmiophilic granules. Furthermore, the granules in such osmium preparations appeared as dull structures, whereas osmiophilic granules show a high index of refraction. Therefore, it seems very probable that the granules demonstrated in the swollen inclusions correspond to the granules observed in the brown inclusion bodies of the Sjövall preparations. Such findings in young Pleuronectes cells lead to the interpretation that the inclusion substance has a granular microstructure even in the early stages of development before the osmiophilic granules appear. The difficulty of demonstrating the "granules of the inclusion substance" in the ordinary preparations of young Pleuronectes lymphocystis cells may be chiefly caused by their dense arrangement. Under favorable fixation and staining conditions, resulting in the brown type of the inclusions in Sjövall preparations, the granules become discernible if inspected with an oil immersion lens of high aperture. In swollen inclusions they are distinctly visible and can easily be demonstrated by simple methods of fixation and staining and with ordinary oil immersion lenses.

The next step was to find out whether such granules of the inclusion substance were still recognizable during the period of appearance of the

"osmiophilic granules." For this task formalin-fixed material of Pleuronectes tumors showing a small number of osmiophilic granules in the surface layer of the inclusion bodies proved unsatisfactory. Only the rather homogeneous "clear type" of the inclusion substance was observed in the osmium preparations, as was shown in Figure 5 of the preceding paper (14). The stage of progressive appearance of the osmiophilic granules is in our collection much better represented by preparations from lymphocystis-diseased perches belonging to the European species *Acerina cernua* and the American species *Stizostedion vitreum*. An experimentally infected young specimen of Stizostedion, which was kept in a laboratory aquarium for 15 weeks, proved especially valuable, since innumerable tumors developed. This material was freshly fixed by various methods, as the fish showed signs of weakness on the 105th day of the infection experiment. From this specimen some Sjövall preparations of relatively small cells were illustrated in Figures 6 and 7 of the preceding paper (14). These pictures did not show granules in the core of the inclusion network. The photomicrograph of a lymphocystis cell in Figure 6 (14) represents a typical picture of the clear type of the inclusion substance. A revised and continued study of the series of Sjövall preparations has, however, led to similar findings, as described in the osmium preparations of young Pleuronectes cells. In addition to the clear type of inclusions the brown type has again been frequently observed, showing quite distinctly, under favorable optic conditions, a granular composition of the inclusion substance.

A section through a lymphocystis cell 200 μ in length, in which the inclusion network is of the brown type, can be noted (Fig. 11). The bars of the network have extended in the upper half of the cell body. The small inclusion area (*a*) is shown in high magnification in Figure 12. The two types of granules are here seen very clearly side by side. The number of osmiophilic granules is still relatively small. They are predominant in the right half of the picture, because here a more superficial plane is in focus. Granules of the inclusion substance, characterized by the lighter osmium staining and a lower index of refraction, are chiefly seen on the left side where a level close to the surface layer is in focus. Among the pale granules (*i*), some dark osmiophilic granules are scattered. It is obvious that the two types of granules are of the same size and that they also resemble one another in their arrangement. They appear rather frequently as dyads and often as tetrads, or arranged in short chains. Sometimes such a chain consists partly of granules of the inclusion substance and partly of osmiophilic granules. Some granules may be found which appear to be intermediate between the two types. The evidence of such Sjövall preparations strongly suggests that the osmiophilic granules originate as peripherally located granules of the inclusion substance which, by a process of transformation, develop a higher index of refraction and a stronger staining reaction with osmic acid. Concerning other structures in Figure 12 it may be pointed out that on the right side, between the groups of osmiophilic granules, a pale brown homogeneous lattice of inclusion substance can be seen which at (*f*) is replaced by clear capsules of a high index of refraction.

Figure 13 shows in a cell from the same tumor a more advanced stage of the development of the inclusion network which here has grown through a larger portion of the cytoplasm. The bar (*b*) of the brown inclusion network is, in Figure 14, demonstrated in high magnification. Whereas at this stage in the periphery a continuous layer of osmiophilic granules (*o*) has developed, the core of the bar shows a mosaic of the more lightly stained granules of the inclusion substance (*i*) which are inbedded into a delicate intergranular mass. In some of the bars, especially in the cortical zone of the cell, the granulated core is replaced by a homogeneous layer of brown inclusion substance or by a strongly refractive unstained framework.

Concerning the occurrence of the clear type and the brown type inclusion substance in the Sjövall preparations of the tumors of this Stizostedion specimen, it can be stated that the clear type is seen chiefly in cortical cells, especially at spots where the covering epithelium is missing, so that here the fixative could exert its quickest and strongest action. In some larger cells the upper half of the cell body near such a surface showed clear inclusions, whereas the inclusion bodies in the opposite half of the cell were all of the brown type. Such observations suggest that the clear type of the inclusion bodies in Sjövall preparations is the result of a fixation phenomenon that reminds one somewhat of the so-called "Überfixation" (Rawitz), which is known to occur frequently in the cortical layer of pieces of organs fixed with Flemming's fluid (cf. 5, Vol. I, p. 477; Vol. II, p. 343).

In the course of the further growth of the lymphocystis cells of perches, which is accompanied by the rapid extension of their inclusion network, a continuous increase in number of the osmiophilic granules is evident. The demonstration of areas of granulated brown inclusion substance in more advanced stages is of interest in determining how this increase occurs. In the Sjövall preparations of the Stizostedion specimen, such areas were still

observed in cells of 350 μ in diameter. Corresponding results were obtained by the study of osmium preparations of Acerina lymphocystis cells. Here such areas have been observed in cells up to 400 μ in diameter. For Acerina this is a rather advanced stage of growth; in this species the tumor cells reach a maximal diameter of only about 700 μ, in contrast to those of Stizostedion which may grow to 1,200 μ. Concerning advanced stages of Pleuronectes cells, areas of granulated brown inclusion substance have been observed in osmium preparations of a tumor containing cells of about 900 μ in diameter.

These findings support the view that such areas may continue, during the growth period of the lymphocystis cells, to serve as germinative layers for the differentiation of osmiophilic granules, provided that in these zones divisions of stem granules balance their numerical reduction brought about by the process of transformation. In this connection, observations in Acerina are of interest in showing that sometimes even an excessive increase in the mass of granulated inclusion substance occurs in medium-sized cells which leads to swelling and finally to the bursting of inclusion bodies. In tumors of an Acerina specimen containing lymphocystis cells up to 320 μ in diameter, it was noted that portions of the inclusion network were subdivided into groups of rather small inclusion bodies which showed various stages of expansion. This is demonstrated (Fig. 15) in a preparation fixed with Meves' fluid and stained with crystal violet after Benda. In the inclusion bodies a basophilic framework of lamellae is reinforced by intensely stained filaments. The lamellae surround a more lightly stained basophilic mass, for which the noncommittal expression "ground substance" may be used. This mass shows a tendency to expand. The small inclusion bodies (*a*) still show the original condition. At (*b*) expanding inclusion bodies are seen; their lamellae have become ruptured. At (*c*) the reinforcing filaments are pushed apart and then break (*d*). Thus, the inclusion bodies finally burst, and the ground substance (*e*) pours into the cytoplasm, mixed with fragments of the broken-down filaments (*f*). As a matter of special interest, it can be seen rather distinctly that the escaping ground substance is composed of fine basophilic granules.

The study of osmium preparations from the same tumor gave the opportunity to analyze the nature of these granules. The Sjövall preparation (Fig. 16) shows that the expanding and finally escaping ground substance chiefly consists of lightly stained granules of the inclusion substance. Osmiophilic granules are seen only in the periphery. On the right side they still mark the original contour of the inclusion body, which in this section has a triangular shape. On the left side the inclusion body has burst, so that here the mass of the granulated inclusion substance has escaped into the cytoplasm. The unstained capsules (*f*) around groups of osmiophilic granules are remnants of the lamellae. Such an expansion and bursting of inclusion bodies with liberation of granules of the inclusion substance has likewise been observed in Stizostedion cells of 450–500 μ.

On the basis of the data obtained for Acerina on the basophilic staining of the granules of the inclusion substance, it appears probable that mosaics of basophilic granules observed in some inclusion lamellae of the experimentally infected young Stizostedion specimen on the 77th and 99th day of the experiment contain granules of the inclusion substance but no osmiophilic granules at this stage (Fig. 17).

DISCUSSION

Mosaics of granules have been described in the intracytoplasmic inclusion bodies of a number of viruses, e.g., in psittacosis (1–4), fowl-pox (6), and in the advanced stages of the inclusions of lymphogranuloma venereum (8). Such granules have been interpreted either as elementary bodies of the viruses or as preceding virus stages which multiply by fission and finally divide into elementary bodies. It is in conformity with these interpretations of the nature of granular structures observed in cytoplasmic inclusions in other virus diseases that the granules of the inclusion substance in the lymphocystis cells are interpreted as developmental stages of the lymphocystis virus. The type of their arrangement, especially their appearance as tetrads or short chains, suggests that the increase in their number is brought about by division of the granules. Apparently, they represent vegetative stages of the virus which participate essentially in the growth of the inclusion bodies and thus increase the virus mass within the host cell. Later, many of the granules of the inclusion substance are transformed into osmiophilic granules.

The bursting of expanded inclusion bodies with the escape of granules of the inclusion substance into the cytoplasm has been demonstrated in Figures 15 and 16 in Acerina cells. Such findings resemble the disintegration of enlarged inclusions with dissemination of elementary bodies which has been described for several macroviruses, especially when cultivated in chick embryos (4, 8). In such cases, virus elements liberated after the breakdown of the host cell are supposed to be the source of the infection of adjacent cells. Certain aspects of the lymphocystis disease may be explained on a

similar basis. In the immediate neighborhood of medium-sized lymphocystis cells newly developed small lymphocystis cells are often found (11, 13). Sometimes a young lymphocystis cell has even been observed within the membrane of a degenerated larger lymphocystis cell (7). In an attempt to interpret such findings, the possibility has to be considered that a new infection by virus units from an outside source may have established itself at the same location as the previous one (11). However, the alternate interpretation, first given by Joseph (7), that infectious agents may have invaded adjacent cells or phagocytes after their development in the larger cells, now gains credence because of the observations in lymphocystis cells of perches showing escape of granules of the inclusion substance into the cytoplasm. There are two possibilities by which the infected cytoplasm of lymphocystis cells might come into direct contact with other cells: (*a*) when the cytoplasm protrudes through the damaged cell membrane; or (*b*) when phagocytes invade a degenerating lymphocystis cell. Both phenomena are frequently observed in lymphocystis tumors.

In contrast to the possible role which granules of the inclusion substance may play in the infection of adjacent cells, the osmiophilic granules apparently represent the stages of the virus which serve to transmit the infection from host to host. Tumors containing either full-grown cells or almost full-grown cells have proved to be very infectious as suspensions of ground cells. In such material, granules of the inclusion substance, if still persisting, would be present in small amounts, whereas the osmiophilic granules would have accumulated as large masses.

It is doubtful whether the osmiophilic granules are capable of increasing their number by division after they have developed by transformation of granules of the inclusion substance. An arrangement of them in dyads, tetrads, or short chains, which has been frequently observed (14), could represent the persisting arrangement of the granules from which they have developed by transformation and which in their turn originated as division products from stem granules.

Hitherto it has not been proved that the infection can be transmitted by an inoculation of the contents of large lymphocystis cells into tissues. An effective method developed by the author for experimental infection consists of forcing a suspension of ground large tumor cells into the lumen of the pharynx by a syringe, either through the mouth or through the opercular clefts (12, 13). This method imitates the presumed natural conditions of the infection. Evidently virus particles become liberated when large lymphocystis cells disintegrate in the water after tumor pieces have been shed or after the death of the fish. Probably such particles may float for some time and perhaps pass through other organisms, until finally some of them are sucked into the pharynx of a fish of the host species via respired water, or possibly are swallowed with food. The storage of masses of osmiophilic granules in large numbers in full-grown lymphocystis cells suggests that these granules are the form in which the infection is transmitted from host to host.

The study of the finer structures of the inclusion bodies in the lymphocystis cells has thus led to the conclusion that in the developmental cycle of the lymphocystis virus two types of granules of elementary size can be distinguished: (*a*) the granules of the inclusion substance which represent vegetative stages of the virus and (*b*) the osmiophilic granules interpreted as virus particles which transmit the infection. For other viruses it has not been shown that elementary bodies, formed as products of the multiplication of the virus within the inclusion bodies, must undergo a change to become transmission stages of the virus. For two reasons one might expect that the developmental cycle of the lymphocystis virus would show some peculiarities. First, in the lymphocystis cells, which hypertrophy to gigantic dimensions, the vegetative phases of the virus, as components of the inclusion bodies, reach a higher level in growth and differentiation than in other viruses. Secondly, with regard to the transmission of the infection from host to host, the lymphocystis virus represents the rare case of a water-borne virus adapted to parasitism in aquatic animals.

The relation of the granules of the inclusion substance to the formation of the framework of the inclusion bodies, which apparently must be considered as an additional vegetative phase of the virus, will be discussed in another paper.

SUMMARY

The formation of the osmiophilic granules, previously interpreted as elementary bodies of the lymphocystis virus (14), in the outer layer of the inclusion bodies of lymphocystis tumor cells is not an abrupt process. In stages preceding the appearance of the osmiophilic granules, a granular composition of the basophilic inclusion substance can be demonstrated in thin layers of typical inclusion bodies by the Sjövall osmium method as well as in swollen inclusions with ordinary methods of fixation and staining. In the osmium preparations the "granules of the inclusion substance" show a slighter staining reaction with osmic acid

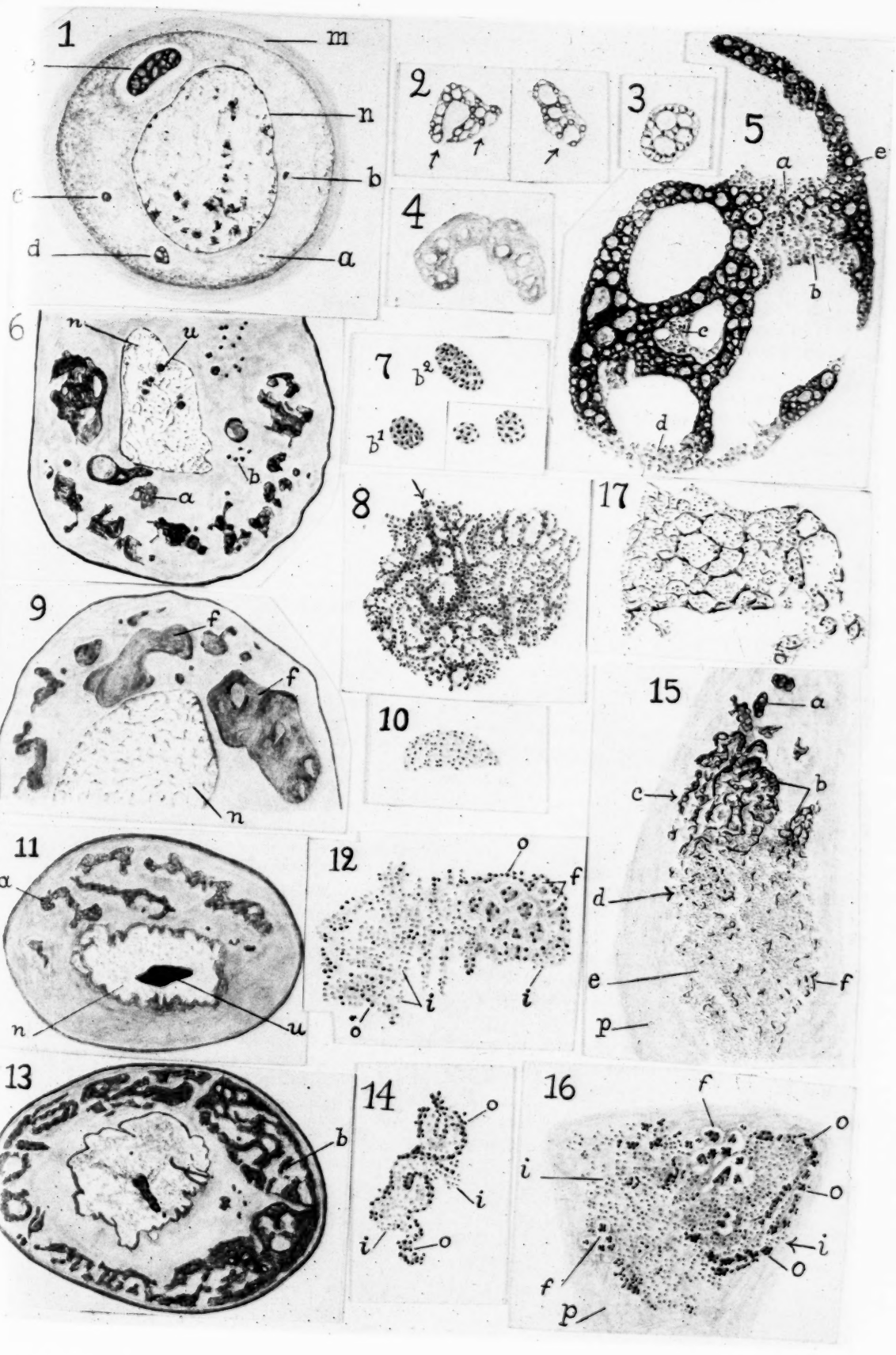

1
m
n
b
c
d
a
2
3
4
5
e
a
b
c
d
6
n
u
b
a
7
b2
b1
8
17
9
f
f
n
10
15
a
c
b
d
e
f
p
11
a
n
u
12
o
f
i
i
o
13
b
14
o
i
i
o
16
f
o
i
o
i
o
f
p

FIG. 1.—Section through a lymphocystis cell of about 40 μ in diameter from an experimentally infected Pleuronectes specimen on the 30th day of the experiment. Fixation with acetified alcohol (absolute alcohol, 9.5 parts; glacial acetic acid, 0.5 parts). Staining with hematoxylin. ×1125. (*a*), (*b*), (*c*), (*d*), (*e*) five stages of the growth of the intracytoplasmic inclusion bodies; (*m*) cell membrane; (*n*) nucleus.

Figures 2–5 show inclusion bodies from lymphocystis cells of 100–200 μ in diameter of the same fish as in Fig. 1, fixed however on the 68th day of the experiment. ×1125.

FIG. 2.—Fixation with acetified alcohol. Staining with hematoxylin. The arrows mark spots where the cortical layer of the inclusions has ruptured.

FIG. 3.—Fixation with fluid of Meves (mixture of 2 per cent aqueous osmic acid, 4 parts; 1 per cent aqueous chromic acid, 15 parts; glacial acetic acid, 3 drops). Staining with safranin and Cajal's mixture of indigo carmine and picric acid.

FIG. 4.—Sjövall's method: fixation with mixture of formalin (1 part) and distilled water (3 parts), staining with 2 per cent aqueous osmic acid for 3 days at 37° C. Inclusion body of the clear type.

FIG. 5.—Sjövall's method. Inclusion body of the brown type which at the areas (*a*), (*b*), (*c*), (*d*) and partly in the bar (*e*) shows granules of the inclusion substance.

Figures 6–10 from a specimen of Pleuronectes caught in September with lymphocystis tumors in which the cells have reached 220–270 μ in diameter and often show swollen inclusion bodies. Fixation with acetified alcohol. Staining with hematoxylin.

FIG. 6.—Section through a lymphocystis cell containing inclusion bodies at an early stage of swelling. (*a*) inclusion body of medium size; (*b*) group of small inclusion bodies; (*n*) nucleus; (*u*) nucleolus. ×240.

FIG. 7.—Four of the small inclusion bodies of group (*b*) of Fig. 6, magnified ×1750. (b^1) and (b^2) inclusions in which a number of the granules of the inclusion substance are arranged as tetrads.

FIG. 8.—The inclusion body (*a*) of Figure 6, magnified ×1750. The arrow points to a tetrad of granules of the inclusion substance.

FIG. 9.—Section through a lymphocystis cell with inclusions showing an advanced stage of swelling. (*f*) inclusions swollen into large fluffy bodies; (n) nucleus. ×240.

FIG. 10.—Small portion of a swollen inclusion body magnified ×1125.

Figures 11–14 from an experimentally infected young specimen of Stizostedion on the 105th day of the experiment. Sjövall's method.

FIG. 11.—Section through a lymphocystis cell (long diameter, 200 μ) in which the inclusion network has extended through one-half of the cell only. (*a*) piece of an inclusion bar; (*n*) nucleus; (*u*) nucleolus. ×240.

FIG. 12.—Piece (*a*) of the inclusion network of Figure 11, magnified ×1750. (*f*) framework capsules; (*i*) granules of the inclusion substance; (*o*) osmiophilic granules.

FIG. 13.—Section through a lymphocystis cell (long diameter 250 μ) in which the inclusion network has extended through a large portion of the cell body around the nucleus. (*b*) piece of the inclusion network. ×240.

FIG. 14.—Piece (*b*) of the inclusion network of Fig. 13, magnified ×1750. (*i*) granules of the inclusion substance; (*o*) osmiophilic granules.

Figures 15 and 16 show bursting inclusion bodies from an Acerina specimen in which the lymphocystis cells have reached diameters of 260–320 μ.

FIG. 15.—Fixation with Meves' fluid. Staining with crystal violet (Benda's method for the staining of mitochondria). ×675. (*a*), (*b*), (*c*), (*d*) successive stages of expansion and bursting of the inclusion bodies; (*e*) mass of granules of the inclusion substance escaping into the cytoplasm; (*f*) fragment of a framework filament; (*p*) cytoplasm.

FIG. 16.—Sjövall's method. ×1125. (*f*) framework capsules; (*i*) granules of the inclusion substance; (*o*) osmiophilic granules; (*p*) cytoplasm.

FIG. 17.—Lamella of an inclusion body from a Stizostedion lymphocystis cell on the 99th day of the infection experiment. Fixation with Flemming's fluid (mixture of 2 per cent aqueous osmic acid, 4 parts; 1 per cent aqueous chromic acid, 15 parts; glacial acetic acid, 1 part). Staining as in Figure 3. ×1125. The lamella shows a network of framework filaments covered by granules interpreted as granules of the inclusion substance.

and a lower index of refraction than the osmiophilic granules. Evidence is presented suggesting that the granules of the inclusion substance multiply by fission and eventually form the osmiophilic granules. Thus, in the developmental cycle of the lymphocystis virus two types of particles of elementary size can be distinguished showing different features in osmium preparations: (*a*) the granules of the inclusion substance, interpreted as vegetative stages which serve for the growth of the virus within the host cells, and (*b*) the osmiophilic granules which are considered to be the virus particles which transmit the infection from host to host. Accumulated in large numbers in advanced lymphocystis cells, they are liberated when the tumor cells disintegrate in the water after extrusion or after the death of the fish.

REFERENCES

1. BEDSON, S. P., and BLAND, J. O. W. A Morphological Study of Psittacosis Virus, with the Description of a Developmental Cycle. Brit. J. Exper. Path., **13**:461–66, 1932.
2. ———. The Developmental Forms of Psittacosis Virus. *Ibid.*, **15**:243–47, 1934.
3. BLAND, J. O. W., and CANTI, R. G. The Growth and Development of Psittacosis Virus in Tissue Culture. J. Path. & Bact., **40**:231–41, 1935.
4. BURNET, F. M., and ROUNTREE, P. M. Psittacosis in the Developing Egg. J. Path. & Bact., **40**:471–81, 1935.
5. EHRLICH, P.; KRAUSE, R.; MOSSE, M.; ROSIN, H.; and WEIGERT, K. Encyclopädie der mikroskopischen Technik. Zweite Auflage. Berlin and Wien: Urban & Schwarzenberg, 1910.
6. GOODPASTURE, E. W. Virus Diseases of Fowls as Exemplified by Contagious Epithelioma (Fowl-Pox) of Chickens and Pigeons. In T. M. RIVERS, Filterable Viruses. London: Baillière, Tindall & Cox, 1928.
7. JOSEPH, H. Untersuchungen über *Lymphocystis* Woodc. Arch. f. Protistenk., **38**:155–249, 1918.
8. RAKE, G., and JONES, H. P. Studies of Lymphogranuloma venereum. I. Development of the Agent in the Yolk Sac of the Chicken Embryo. J. Exper. Med., **75**:323–38, 1942.
9. WEISSENBERG, R. Über infectiöse Zellhypertrophie bei Fischen (Lymphocystiserkrankung), pp. 792–804. Sitzungsber. Kgl. Preuss. Ak. Wiss., phys.-math. Cl., Berlin, 1914.
10. ———. Lymphocystisstudien (infectiöse Hypertrophie von Stützgewebszellen bei Fischen). I. Die reifen Geschwülste bei Kaulbarsch und Flunder. Lymphocystisgenese beim Kaulbarsch. Arch. f. mikr. Anat., **94**:55–134, 1920.
11. ———. Lymphocystiskrankheit der Fische, pp. 1344–80. In S. v. PROWAZEK and W. NÖLLER, Handbuch der pathogenen Protozoen, **3**, Lief. 9. Leipzig: J. A. Barth, 1921.
12. ———. Studies on Virus Diseases of Fish. III. Morphological and Experimental Observations on the Lymphocystis Disease of the Pike Perch, *Stizostedion vitreum*. Zoologica, New York Zool. Soc., **24**:245–54, 1939.
13. ———. Studies on Virus Diseases of Fish. IV. Lymphocystis Disease in Centrarchidae. *Ibid.*, **30**:169–84, 1945.
14. ———. Studies on Lymphocystis Tumor Cells of Fish. I. The Osmiophilic Granules of the Cytoplasmic Inclusions and their Interpretation as Elementary Bodies of the Lymphocystis Virus. Cancer Research, **9**:537–42, 1949.
15. WOODCOCK, H. M. Note on a Remarkable Parasite of Plaice and Flounders. Rep. Lancashire Sea-fisher. Lab. for 1903. Liverpool, 1904.

The Metabolism of Thyroxine in C57 Mice and in C3H Mice with and without Mammary Tumors*

J. Gross† and S. Schwartz

(*Department of Anatomy, McGill University, Montreal, Canada*)

There have been varied reports in the literature which indicate that the thyroid hormone may play a role in tumor development. However, it has been recently shown that spontaneous mammary tumor development in C3H mice may be inhibited by the administration of anti-thyroid drugs (2, 12, 17). With the availability of "tracer" amounts of I^{131}-labeled thyroxine (5), it seemed of interest to determine and compare the metabolism of this hormone in female C3H and C57 mice, which show a high and a low incidence, respectively, of mammary carcinoma.

about 0.02 μg. of thyroxine with a radioactivity content of 0.2 μc. of I^{131}.

Three separate experiments were carried out, as follows. In the first experiment, three groups of animals were used, consisting of eight female C57 mice, eight C3H mice without tumors, and eight C3H tumor-bearing mice, respectively. The first two groups were approximately 6 months old and weighed 22 ± 2 gm. None of the C3H animals of the second group showed any grossly visible tumor nodules at autopsy. The age of the tumor-bearing animals averaged about 9 months, and they had

TABLE 1

THE DISTRIBUTION OF LABELED L-THYROXINE AS PERCENTAGE OF THE RECOVERED DOSE OF RADIOACTIVITY PRESENT IN THE ORGANS AND EXCRETA OF C57 AND C3H MICE

	C57		C3H		C3H TUMOR-BEARING	
	2 Hours after injection Mean±s.e.	24 Hours after injection Mean±s.e.	2 Hours after injection Mean±s.e.	24 Hours after injection Mean±s.e.	2 Hours after injection Mean±s.e.	24 Hours after injection Mean±s.e.
Plasma	13.94±0.99	2.34±0.49	12.01±0.77	3.44±0.34	10.66 ±0.90	3.51 ±0.56
Liver	21.20±0.89	4.88±0.24	26.76±0.67	10.50±0.86	28.90 ±1.02	9.14 ±0.37
Intestinal tract	15.11±1.07	3.99±0.51	13.01±0.61	5.80±0.78	9.26 ±1.52	6.54 ±0.40
Kidney	2.75±0.18	0.73±0.073	3.66±0.12	1.24±0.17	2.97 ±0.12	1.01 ±0.054
Skin	9.84±0.62	4.47±0.51	12.48±0.50	7.51±0.97	10.50 ±0.51	7.75 ±0.78
Carcass	32.64±1.05	8.60±0.58	30.73±0.51	14.26±1.80	25.46*±1.45	13.79*±1.26
Feces	0.13±0.047	43.1 ±2.0	0.04±0.014	34.8 ±5.3	0.04 ±0.14	41.1 ±1.6
Urine	4.11±0.47	31.0 ±1.0	0.47±0.053	20.7 ±2.8	1.05 ±0.48	17.0 ±2.3

* Including percentage of radioactivity in the tumor.

The results obtained seemed to indicate that the thyroid hormone may be a contributing influence in the development of mammary carcinoma. Furthermore, evidence was obtained that the metabolism of thyroxine in mammary tumor tissue may be different from that in the normal tissues examined.

METHODS

Radiothyroxine was prepared from the plasma of NaI^{131}-treated rats as previously described (5). The amounts injected were estimated to contain

tumors which varied in size from 80 to 5,700 mg. (average 2,500 mg.). The eight animals in each group were divided into two sub-groups of four animals, which were sacrificed at 2 and 24 hours after the intravenous injection of the radiothyroxine. The animals were exsanguinated under ether anesthesia, and the organs were rapidly removed and weighed. The remainder of the animal was weighed and labeled as carcass. Any large accumulations of old blood or necrotic material were removed from the tumor and added to the carcass sample. The radioactivity measurement of the organs and excreta (and in some cases butanol fractionation into thyroxine-like and nonthyroxine iodine) was carried out as previously described (5), and thyroxine was detected by paper chromatography (7).

* This work was supported by a grant from the National Cancer Institute of Canada to Dr. C. P. Leblond.

† Present address: The National Institute for Medical Research, Mill Hill, London, N.W. 7, England.

Received for publication November 18, 1950.

The volume of solution injected was small (0.1 ml.) and subject to error in measurement. However, since the radioactivity of the entire animal was measured, the data were calculated on the basis of the recovered dose (Table 1, Chart 1). The average recovery was 95 per cent of the prepared standard, which itself was subject to a similar measurement error.

The second experiment was carried out on three tumor-bearing C3H mice, which were injected subcutaneously with labeled thyroxine. Four hours after injection the animals were anesthetized with ether, and a tumor biopsy was taken. Twenty hours later the mice were sacrificed, the remaining tumor tissue and some organs being examined for radioactivity content (Table 2).

TABLE 2

THE CONCENTRATION OF RADIOACTIVITY IN MAMMARY TUMOR TISSUE AT 4 HOURS, AND MAMMARY TUMOR AND OTHER TISSUES AT 24 HOURS AFTER THE SUBCUTANEOUS ADMINISTRATION OF 0.02 μG. OF LABELED L-THYROXINE INTO C3H MICE

	Counts/100 secs/mg tissue / Counts/100 secs/mg body weight		
	Animal A	Animal B	Animal C
Tumor biopsy at 4 hrs.	0.52	0.42	0.43
Tumor at 24 hr.	0.56	0.54	0.58
Liver	1.31	1.17	1.15
Plasma	1.26	1.28	1.00
Kidney	0.70	0.57	0.69
Mammary gland	0.48	0.40	0.42
Carcass	0.41	0.46	0.35

As a sequel to the above experiment, six groups, each containing five tumor-bearing C3H females, were injected subcutaneously with labeled thyroxine and sacrificed after intervals of 1, 2, 6, 12, 24, and 72 hours, respectively. The total and, in some cases, the butanol-soluble radioactivity were determined in the tumors, mammary glands, livers, plasmas, ovaries, carcasses, and excreta (Chart 2, 3, Table 3).

RESULTS

The over-all distribution of radioactivity is shown in Table 1. In all groups, 2 hours after the injection, most of the radioactive thyroxine had been removed from the plasma and could be found mainly in the liver, gastrointestinal tract, carcass, and skin. By 24 hours, most of the injected material had left the body and was present in the feces and to a lesser extent in the urine. However, the rate of excretion was more rapid in the C57 animals (74 per cent of administered radioactivity in 24 hours), as compared to that occurring in either of the C3H groups (i.e., 55 per cent and 58 per cent). The increase was due to a greater urinary excretion of radioactivity by the C57 animals, there being no significant difference in the 24-hour fecal radioactivities between the three groups. As well, the proportion of the fecal iodine identifiable as thyroxine (7) was roughly the same (about 50 per cent) in all groups.

The lower rate of thyroxine elimination in the C3H strain was paralleled by a less rapid decline in the radioactivity of the individual organs and tissues (Table 1, Chart 1). By subjecting the data in Chart 1 to analysis of variance, it could be shown that the decrease of I^{131} concentration between 2 and 24 hours in the plasma, liver, intestinal tract, ovary, and carcass of the nontumor-bearing C3H mice was significantly slower than in the corresponding organs of the C57 mice. In addition, at 2 hours after injection, the concentration of I^{131} in the livers of the C3H animals was significantly greater, and the intestinal I^{131} significantly smaller, than in the corresponding organs of the C57 animals.

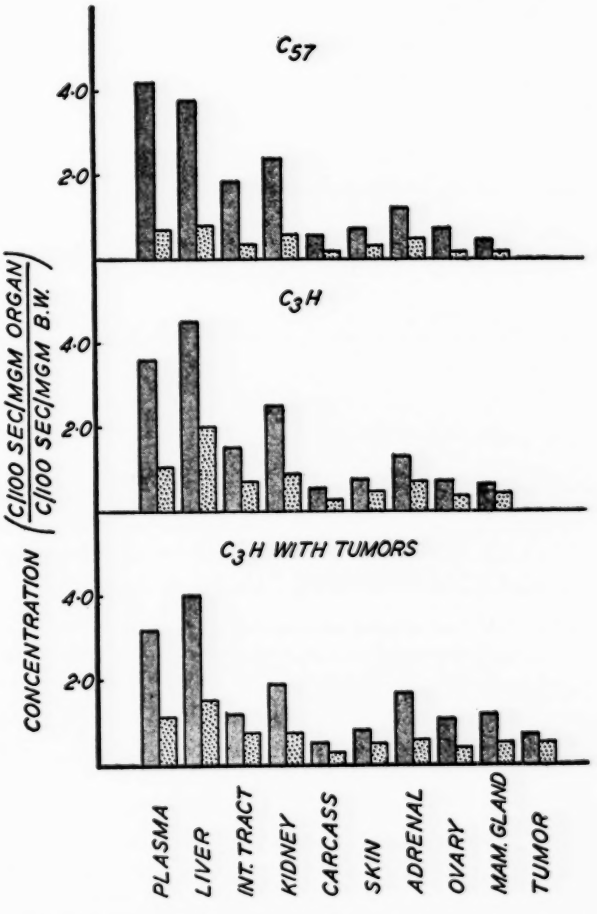


CHART 1.—The distribution of the I^{131} concentration in various organs and tissues after the intravenous injection of 0.02 μg. of labeled L-thyroxine. Each column represents the average of the values from four animals. The stippled columns represent the values at 2 hours, the dotted columns at 24 hours after injection.

Analysis of variance of the data at 2 and 24 hours showed no significant difference between the two groups of C3H mice in the decrease of organ radioactivity (Table 1, Chart 1).

In the tumor (Chart 1), the concentration of radioactivity was quite low relative to that of the other tissues examined. However, it may be noted that the relative decrease between the 2- and 24-hour concentrations appeared to be considerably less than in the noncancerous tissue. To investi-

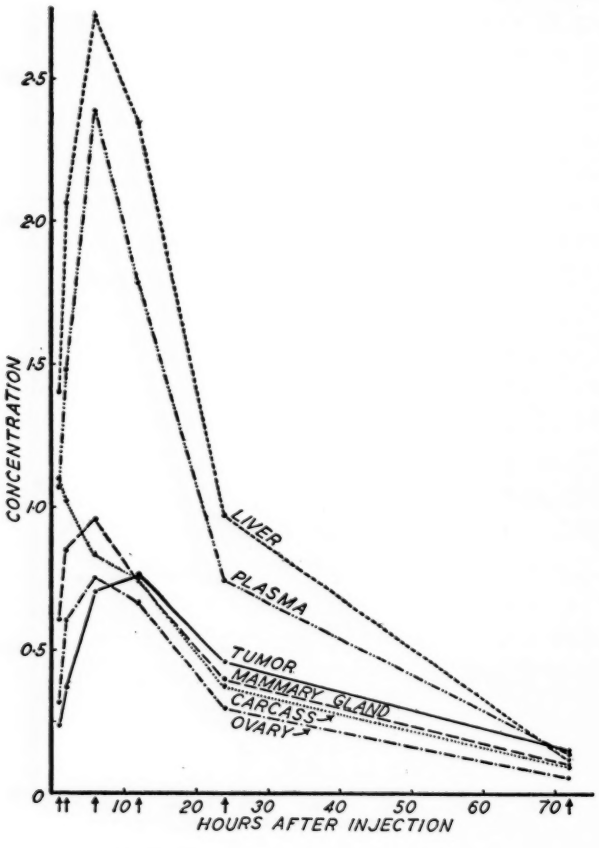


CHART 2.—The variation of I^{131} concentration with time in the organs of C3H tumor-bearing mice, following the subcutaneous administration of 0.02 μg. of radiothyroxine. Each point represents the average of the values from 5 animals. Concentration is expressed in the same units as in Chart 1.

gate this point, the second and third experiments were carried out and gave the following results.

When the concentration of labeled material present in the tumor was determined at 4 and 24 hours after the subcutaneous injection of radioactive thyroxine (Table 2), in all the animals there was a greater concentration at the latter time. Furthermore, at the time of sacrifice the tumor I^{131} concentration exceeded that of normal mammary tissue and of the carcass but was less than the concentration found in the plasma, liver, or kidney.

These points were confirmed and amplified in the subsequent experiment (Chart 2), from which it may be seen that after a *subcutaneous* injection of a tracer dose of radiothyroxine a peak concentration of I^{131} occurs at 6 hours in the liver, plasma, mammary gland, and ovary; and at 12 hours

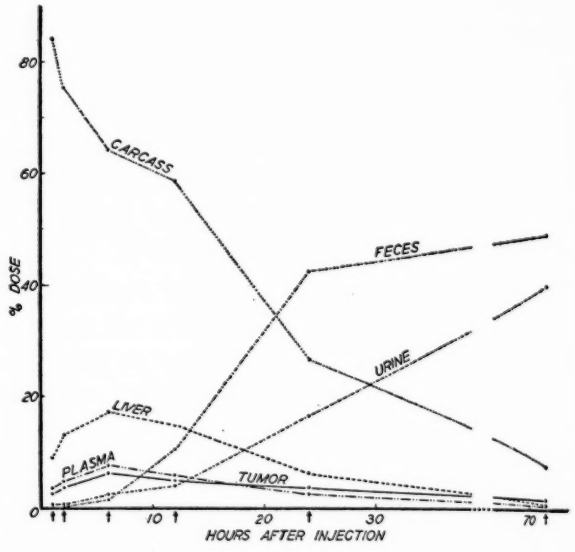


CHART 3.—The distribution of I^{131} as percentage of the administered radioactivity in C3H tumor-bearing mice at various times after the subcutaneous administration of 0.02 μg. of radiothyroxine. Each point represents the average of the values from five animals.

TABLE 3

THE PERCENTAGE OF BUTANOL-SOLUBLE RADIOACTIVITY IN SEVERAL TISSUES AFTER THE SUBCUTANEOUS ADMINISTRATION OF 0.02 μG. OF LABELED L-THYROXINE

	Hours after injection			
	1	2	6	12
Plasma	77	93 (88–98)	94* (39–95)	94* (91–96)
Tumor	78	72	75* (56–87)	69* (65–74)
Mammary gland	67	64	65	67

* The range of values is indicated in parentheses below the average. In these cases, individual samples from the five animals were fractionated and the mean calculated.

The other values represent the results of the fractionation of the pooled samples of the five animals in the group as, in these cases, the low radioactivity of the individual tissues prevented individual determinations. At 24 and 72 hours the radioactivity of the pooled sample was too low for fractionation.

in mammary tumor tissue. Also, after 12 hours, the concentration of labeled material in the tumor exceeds that found in the carcass, mammary gland, and ovary, and at 72 hours is equal to that of the liver. On fractionation with *n*-butanol, most of the tumor I^{131} is found in the thyroxine-like fraction (Table 3), the proportion being somewhat less

than that found in the plasma and somewhat more than that found in the mammary gland.

The over-all behavior of the injected dose is shown in Chart 3, from which it may be seen that the thyroxine iodine is excreted rather rapidly from the body, appearing primarily in the feces and to a lesser extent in the urine. This excretion follows an exponential curve and gives a thyroxine half-life of about 20–22 hours in these animals. The liver, plasma, and tumor contain not inconsiderable amounts of labeled material, although the carcass, exclusive of these tissues, contains the greater proportion of I^{131} at all time intervals.

DISCUSSION

The metabolism of tracer doses of labeled thyroxine in rodents appears to be indistinguishable from that of the endogenously formed thyroid hormone (5, 6). In the present series, the weight of thyroxine injected (0.02 μg.) was assumed to be sufficiently small so as to behave as a tracer substance and thus to indicate the normal metabolism of thyroid hormone in the two strains of mice.

In comparing the results in the C3H and C57 animals (Table 1, Chart 1), the most striking difference was the greater urinary excretion of the labeled material from the latter strain. Since most of the urinary radioactivity occurs as *iodide ion* (4, 6), the metabolism (deiodination) of thyroxine is more rapid in the C57 mice. The primary factor in this strain difference in thyroxine metabolism would appear to be a strain difference in liver function, since this organ has been shown to play a major role in the excretion, degradation (4, 5), and detoxification of thyroxine (3). This is borne out by the greater concentration of radioactivity in the C3H livers at 2 hours after injection, associated with a much slower decrease in I^{131} concentration between 2 and 24 hours, than is found in the corresponding organs of the C57 animals (Chart 1).

The net result of this metabolic difference is that the iodine of a thyroxine molecule has a longer mean duration in the body of a C3H, as compared to a C57 mouse. From the data, this duration can be calculated (cf. [5]) to be 30 hours and 17 hours, respectively. However, the difference in the thyroxine secretion rates is not as great, being 6.0 and 7.0 μg of DL-thyroxine/100 gm body weight/day, respectively (15).[1] From these figures, it may be calculated[2] that the total body thyroxine (exclusive of thyroid thyroxine) is 1.65 μg. in the C3H, and 1.04 μg. in the C57 animals used in this experiment. That is, the concentration of thyroid hormone in the body is higher in the C3H than in the C57 mice.

This increased concentration and duration of thyroxine in the C3H mouse may be a factor in mammary tumorigenesis. Thus, in mice, unlike rats (16), thyroxine appears to increase the responsiveness of mammary tissue to gonadal hormones (9, 10). Conversely, anti-thyroid drugs inhibit mammary gland growth and tumor formation (2, 12, 17) without themselves exerting a direct effect on mammary tissue (9)—i.e., by causing a deficiency of thyroid hormone. It is possible, then, that the increased level and duration of thyroxine in C3H mice may increase the responsiveness of mammary tissue to gonadal hormones and thus facilitate the appearance of mammary tumors.

The thyroxine metabolism in C3H mice does not appear to be affected either by the size or presence of a mammary tumor, since no differences in distribution or correlation with tumor weight could be demonstrated. It is likely that the skin I^{131} concentration correlation with tumor weight after radiothyroglobulin administration, observed by Scott *et al.* (13), was due to some iodinated compound other than thyroxine. Indeed, the same authors also failed to find any similar correlation with gastrointestinal tract radioactivity after radiothyroxine administration (14).

With regard to thyroxine metabolism in tumor tissue, if the peak values of the curves in Chart 2 approximate the true peak values, it is evident that the tumor tissue showed a slower rate of entry and exit[3] of radioactivity than did the normal tissues. This would represent a slower turnover of thyroxine or closely related metabolites, since most of the tumor radioactivity was butanol-soluble (Table 3). It is unlikely that this effect is due to localization of the radioactive material in the necrotic tissue of the tumor, as has been indicated in the case of certain halogenated dyes (17), since no correlation could be demonstrated between tumor radioactivity and a rough estimate of the percentage of tumor necrosis in the individual animals. Thus, the slower turnover of thyroxine iodine appears to be a function of active tumor tissue. The

[1] F. W. Smith, personal communication.

[2] If it is assumed that thyroid hormone metabolism is in a steady state over short periods of time (1), (that is, that the rate of formation is equal to the rate of loss from the body), it follows that if, in the case of the C57 animal, the total body thyroxine (exclusive of that of the thyroid) is renewed in 17 hours and the rate of hormone secretion is 7.0 μg/100 gm of body weight/day (i.e., 1.47 μg/day for a 21-gm. animal), then the total body thyroxine content is equal to 1.04 μg. (i.e., $\frac{17}{24} \times 1.47$). In the same way, the body thyroxine for the 22-gm. C3H mouse is calculated to be 1.65 μg.

[3] From the curves in Chart 2, it may be seen that tumor concentration of radioactivity decreases to half its peak value in 24 hours, while the plasma radioactivity does so in 13 hours and the other normal tissues in 15 hours.

mechanism and significance of this phenomenon are unknown. However, in view of all the well known effects of thyroid hormone on oxidative metabolism, it may be a factor in the metabolic effects peculiar to tumor tissue (cf. LePage [8]).

SUMMARY

1. Tracer doses of thyroxine labeled with I^{131} were injected intravenously into C57, C3H, and C3H tumor-bearing female mice and their distribution determined at 2 and 24 hours after administration.

In a second series of experiments, tracer doses of radiothyroxine were administered subcutaneously to tumor-bearing C3H mice, and the I^{131} content of a tumor biopsy specimen at 4 hours was compared to the tumor and organ radioactivities at 24 hours after injection.

In a third experiment, the metabolism of radiothyroxine was followed in groups of tumor-bearing C3H animals at 1, 2, 6, 12, 24, and 72 hours after the administration of the labeled material.

2. It was found that there was a less rapid rate of I^{131} excretion in the C3H animals than in the C57 group; the mean life of thyroxine iodine in the body was 30 and 17 hours, respectively. Similarly, the I^{131} of the plasma, liver, intestinal tract, and ovary decreased more slowly, and the rate of conversion of thyroxine to its metabolites was less rapid in the tumor-susceptible strain. From the data, it was calculated that the body content of thyroxine in the C3H was $1\frac{1}{2}$ times greater than in the C57 mice. The possible relationship of these findings to the hormonal influences in mammary tumor development are discussed, and it is suggested that the thyroid hormone may be a contributing factor.

3. The presence or the variation in size of the mammary tumor had no demonstrable effect on the metabolism of thyroxine

4. The radioactivity of the tumor tissue declined about $1\frac{1}{2}$ times less rapidly than did that of the normal tissues and was predominantly present in butanol-soluble form (i.e., as thyroxine or closely related compounds).

The data would indicate that the delay in thyroid hormone turnover is a function of the active tumor tissue, and it is suggested that this may be a factor in the metabolic processes peculiar to tumor tissue.

ACKNOWLEDGMENTS

The authors are indebted to Dr. C. P. Leblond for encouragement in the course of the experimental work and for criticism of the manuscript; and to Miss Jeanne Cambron for extensive technical assistance.

The authors are also indebted to Dr. W. L. M. Perry and Miss G. Davies of the National Institute for Medical Research, London, England, for advice in, and the carrying out of, the statistical analysis.

REFERENCES

1. Dougherty, J.; Gross, J.; and Leblond, C. P. Steady State of the Thyroidal Iodine Endocrinology (in press).
2. Dubnik, C. S.; Morris, H. P.; and Dalton, A. J. Inhibition of Mammary Tumor Formation in Female C3H Mice Following the Ingestion of Thiouracil. J. Nat. Cancer Inst., **10**:815–39, 1950.
3. Grad, B., and Leblond, C. P. Effect of Thyroxine on Oxygen Consumption and Heart Rate, Following Bile Duct Ligation and Partial Hepatectomy. Am. J. Physiol., **162**:17–23, 1950.
4. Gross, J., and Leblond, C. P. Distribution of a Large Dose of Thyroxine Labeled with Radioiodine in the Organs and Tissues of the Rat. J. Biol. Chem., **171**:309–20, 1947.
5. ———. Metabolism of the Thyroid Hormone in the Rat as Shown by Physiological Doses of Labeled Thyroxine. *Ibid.*, **184**:489–500, 1950.
6. ———. Metabolites of Thyroxine. Proc. Soc. Exper. Biol. & Med. **76**:686–89, 1951.
7. Gross, J.; Leblond, C. P.; Franklin, A. E.; and Quastel, J. H. Presence of Iodinated Amino Acids in Unhydrolyzed Thyroid and Plasma. Science, **111**:605–8, 1950.
8. LePage, G. A. A Comparison of Tumor and Normal Tissues with Respect to Factors Affecting the Rate of Anaerobic Glycolysis. Cancer Research, **10**:77–88, 1950.
9. Mixner, J. P. The Influence of Thiouracil on Mammary Lobule-alveolar Growth in Mice. J. Dairy Sc., **30**:578, 1947. "Abstr.," Obst. Dairy Sc., **9**:189, 1947.
10. Mixner, J. P., and Turner, C. W. Influence of Thyroxine upon Mammary Lobule-alveolar Growth. Endocrinology, **31**:345–48, 1942.
11. Moore, F. D.; Tobin, H.; and Aub, J. C. Studies with Radioactive Di-azo Dyes. III. The Distribution of Radioactive Dyes in Tumor-bearing Mice. J. Clin. Investigation, **22**:161–68, 1943.
12. Morris, H. P.; Dubnik, C. S.; and Dalton, A. J. Effect of Prolonged Ingestion of Thiourea on Mammary Glands and Mammary Tumors in Adult C3H Mice. J. Nat. Cancer Inst., **7**:159–69, 1946.
13. Scott, K. G.; Bostick, W. L.; Shimkin, M. B.; and Hamilton, J. G. Distribution of Globulin-bound Thyroid Fractions of Iodine in Normal and Tumorous Animals after Intravenous Administration Using Radioiodine as a Tracer. Cancer, **2**:692–96, 1949.
14. Scott, K. G., and Stone, R. S. Tumor-Host Studies. II. Increased Concentration of Tagged Iodotyrosines in the Gastrointestinal Tract of Rats Bearing Tumors. Cancer, **3**:722–24, 1950.
15. Smith, F. W., and Gardner, W. U. Thyroid Secretion Ratio in Different Stocks of Mice. Anat. Rec., Suppl., **103**:506, 1949.
16. Trentin, J. J.; Hurst, V.; and Turner, C. W. Thiouracil and Mammary Growth. Proc. Soc. Exper. Biol. & Med., **67**:461–63, 1948.
17. Vasquez-Lopez, E. The Effects of Thiourea on the Development of Spontaneous Tumors in Mice. Brit. J. Cancer, **3**:401–14, 1949.

The Nature of the Fatty Acids of Rats Growing Walker Carcinoma 256*†

FRANCES L. HAVEN, W. R. BLOOR, AND CHALLISS RANDALL

(Department of Biochemistry, University of Rochester School of Medicine and Dentistry, Rochester, N.Y.)

The bodies of rats bearing Walker carcinoma 256 become depleted of fat, while the lipids of the blood plasma increase markedly (10). In the present experiments the nature of the fatty acids of tumor, of host rats, and of pair-fed control rats without tumors was studied in order to interpret the changes in lipid metabolism occasioned by tumor growth.

MATERIALS AND METHODS

Diets.—Male Wistar rats were placed on a semi-synthetic diet at weaning, and the food consumption of each was measured. A week later each rat was paired with one of equal body weight and food consumption. At this time one rat of each pair was designated as the experimental rat which would receive a tumor transplant; the other rat, his pair-fed control, consumed the amount of diet eaten by the experimental rat. Six days after pairing, the experimental rat alone received a subcutaneous transplant of Walker carcinoma 256. Two different diets were used: Diet 262 (16) contained 21.3 per cent Crisco[1] by weight; the other, Diet 290C (19), contained 49.4 per cent elaidin[2] by weight. The elaidin was substituted for all the carbohydrate of the diet in order to minimize the synthesis of fatty acids from carbohydrate and thereby force elaidic acid into the tissues. A test rat that ate the elaidin diet *ad libitum* for 2 months prior to the experiment had an entirely normal growth curve; no abnormalities were revealed at autopsy.

* This investigation was supported by a research grant from the National Cancer Institute, of the National Institutes of Health, Public Health Service; by a grant from the Jane Coffin Childs Memorial Fund for Medical Research; and by a grant from the Fluid Research Fund of the University of Rochester School of Medicine and Dentistry.

† Presented in part at the Meeting of the American Association for Cancer Research, Inc., Cleveland, April, 1951.

[1] The Crisco had an iodine number of 32–33; solid acids comprised 50 per cent of the fatty acids.

[2] The elaidin, prepared by passing nitrogen trioxide into olive oil (19), was white, melted at 38°–39° C., and had an iodine number of 69–70; 95 per cent of the fatty acids consisted of solid acids.

Received for publication February 18, 1951.

Preparation of tissues for analysis.—Rats to be sacrificed were anesthetized with diethyl ether. Blood for analysis was obtained from the hearts of the animals on Crisco; the animals on elaidin were not bled but were allowed to asphyxiate. The gastrointestinal tract of each rat was removed, slit open, washed out, and ground with the carcass. Liver and tumor (including any large metastases) were removed, weighed, and minced. Carcass,[3] liver, and tumor, respectively, were frozen and dried in the frozen state (lyophilized). The dry material was weighed, ground, and mixed; duplicate portions of each tissue approximating 1 gm. were accurately weighed.

Extraction of lipids.—Each weighed portion of dry carcass, liver, or tumor, wrapped in porous filter paper, was suspended beneath the drip of a reflux condenser connected by a ground glass joint with a flask on a hot plate. Each sample was hot-extracted for 5 and 3 hours, respectively, with fresh portions of a 3:1 mixture of 95 per cent ethyl alcohol and diethyl ether, then for two periods of 3 hours with a 1:1 mixture of chloroform and methyl alcohol.

ANALYSES

1. *Total lipid.*—Analysis for total lipid of the blood plasma was carried out by a method previously described (2, 10). The extracts from each sample of dry tissue were combined and the solvent removed under partial vacuum at a temperature not exceeding 50° C. The residue was extracted with a 6:1 mixture of purified petroleum ether and redistilled chloroform and the extract washed with 50 per cent ethyl alcohol. The solution of lipids was transferred to a weighed flask, the solvent removed under nitrogen, and the flask and contents dried to constant weight.

2. *Saponification of lipid.*—To the weighed sample of total lipid dissolved in 3:1 ethyl alcohol-diethyl ether was added approximately 5 drops of saturated aqueous sodium hydroxide per 0.1 gram of lipid. The flask was covered with a watch glass and the contents heated on the hot plate to sticky

[3] By carcass is meant the whole rat minus tumor and liver.

dryness. The residue was transferred to a 50-ml. centrifuge tube by means of 50 per cent ethyl alcohol and the volume brought to 20 ml. The solution was thoroughly extracted with two separate portions of 6:1 petroleum ether-chloroform solution and the extracts of steroids discarded. The alcohol solution was then acidified to Congo Red with 6 N hydrochloric acid and the fatty acids extracted with at least two portions of purified petroleum ether. The extracts were combined in a volumetric flask, the solution made to volume, and aliquots containing about 3 mg. of fatty acids taken for determination of amount (1) and iodine number (23).

3. *Separation of solid and liquid fatty acids.*—The removal of alcohol-insoluble material consisting of unsaponifiable phospholipid and the separation of solid and liquid fatty acids were carried out as described by Sinclair (18, 19). Iodine numbers were determined on each fatty acid fraction (23). The sum of each solid and corresponding liquid acid fraction equalled from 90.2 to 106.8 per cent (median = 99.9 per cent) of the known amount of purified, mixed fatty acid taken for the separation.

EXPERIMENTS AND RESULTS

Rats on Crisco diet.—Table 1 shows the total lipid and fatty acid content, the solid and liquid fatty acids expressed as percentage of total fatty acids, and the iodine number of each fatty acid fraction for carcass and tumor and for pair-fed control rats (see Table 3 for mean values). The carcass of each tumor-bearing rat contained less fatty acid than the pair-fed control; the decrease was more pronounced in rats with tumors comprising over 8 per cent of the body weight. Accompanying the decrease in carcass fatty acids was an increase in total lipid of the blood for each tumor-bearing rat as compared to his pair-fed control. In considering the data of Table 1, attention will be focused on the seven rats with tumors comprising 8 per cent or more of the total body weight, since they showed more pronounced effects of the tumor on the host.

The fatty acid composition of the *tumor* differed qualitatively and quantitatively from that of both the host and control rat. Except in the two rats with large, older tumors where carcass fatty acids were low, the fatty acids made up a much smaller percentage of the dry residue of the tumor than of the carcass. The percentage of solid acids in the tumor was significantly lower with a lower iodine number, while the percentage of liquid acids was correspondingly higher and the iodine number higher than in the carcass of the tumor-bearing rat.

The fatty acid composition of the *host rat* was qualitatively similar to that of the control rat,

TABLE 1

THE FATTY ACID CONTENT OF TUMOR-BEARING (E) AND OF PAIR-FED (C) RATS ON CRISCO DIET

		Carcass									Tumor						
		Blood plasma					Solid acids		Liquid acids					Solid acids		Liquid acids	
	Days after transplant	Total lipid mg. per cent		Diff. E−C	Total lipid per cent dry res.	F.A. per cent dry res.	Per cent F.A.	I.N.	Per cent F.A.	I.N.	Tumor per cent B.W.*	Total lipid per cent dry res.	F.A. per cent dry res.	Per cent F.A.	I.N.	Per cent F.A.	I.N.
E	7	306	+	68	32.5	25.0	42.6	51.3	54.3	107.1	0.3			no analyses			
C		238			42.4	34.4	48.8	62.6	51.0	114.5							
E	10	533	+	143	41.2	33.7	44.2	56.6	56.7	106.4	1.0	†		45.9	48.8	59.5	116.0
C		390			46.6	38.5	47.2	56.6	54.3	108.2							
E	15	600	+	319	21.6	15.1	46.2	54.6	57.0	111.1	12.8	16.6	9.8	43.5	44.4	63.3	121.7
C		281			39.8	32.8	39.6	44.7	62.5	107.3							
E	17	1,500	+1,268		17.0	11.9	46.3	48.1	55.5	118.7	25.1	13.8	7.6	34.6	26.7	68.2	138.0
C		232			41.7	34.7	42.4	45.9	58.8	107.3							
E	19	290	−	51	31.4	26.0	55.6	63.9	46.7	112.4	8.3	14.2	7.3	30.5	26.0	66.9	135.5
C		341			48.3	42.0	48.0	57.0	54.1	107.1							
E	22	435	+	220	20.8	16.7	48.7	60.5	51.1	112.6	18.6	13.0	7.0	33.7	24.6	67.4	129.0
C		215			53.4	46.5	49.1	51.2	53.2	106.4							
E	26	470	+	129	28.2	23.6	48.6	54.9	52.6	108.4	16.4	15.9	8.7	35.1	28.9	64.6	135.9
C		341			34.7	30.1	40.8	47.9	60.6	106.4							
E	28	300	+	42	7.7	3.6	32.5	28.7	63.1	145.4	36.6	13.3	6.7	33.1	27.1	68.4	126.6
C		258			39.7	32.7	35.8	42.7	58.9	107.1							
E	30	550	+	270	8.0	3.6	36.8	27.2	63.8	138.0	23.8	13.7	5.7	27.1	26.0	68.7	156.0
C		280			47.3	40.9	41.6	49.0	58.7	106.7							

* B.W. = Body weight of rat plus tumor.
† No dry weight obtained.
F.A. = fatty acids.

except for the iodine numbers of the liquid fatty acids which were significantly higher in rats with tumors. The percentage and iodine numbers of the solid fatty acids tended to be higher in rats with tumors, with the exception of the last two rats with larger older tumors where the situation was reversed.

The livers of tumor-bearing and control rats on the Crisco diet showed no significant differences in content of total lipid or in fatty acid composition. However, the water content of the liver, expressed as the percentage of either wet tissue or fat-free wet tissue, was significantly higher in rats with tumors than in control rats, as observed for acids higher with higher iodine numbers than in the controls.

The average value of 16.7 per cent of the fat-free dry residue for total lipid of the livers of tumor-bearing rats was significantly less than 19.4 per cent for the controls. The water content of the livers of the experimental rats was not elevated. This terminal decrease in total lipid parallels the premortal decrease in nitrogenous substances of the liver found by Sherman *et al.* (15).

A summary of the mean values for the fatty acid data of Tables 1 and 2 is presented in Table 3. The statistical analysis (5) of the data is indicated. It will be noted from this table that in the Crisco

TABLE 2

THE FATTY ACID CONTENT OF TUMOR-BEARING (E) AND OF PAIR-FED (C) RATS ON ELAIDIN DIET

		Carcass							Tumor							
				Solid acids			Liquid acids					Solid acids			Liquid acids	
	Days after transplant	Total lipid per cent dry res.	F.A. per cent dry res.	Per cent F.A.	I.N.	Elaidic per cent F.A.	Per cent F.A.	I.N.	Tumor per cent B.W.*	Total lipid per cent dry res.	F.A. per cent dry res.	Per cent F.A.	I.N.	Elaidic per cent F.A.	Per cent F.A.	I.N.
E	22	11.5	8.1	56.9	58.2	36.8	43.0	128.5	23.1	10.7	6.3	46.4	56.9	29.3	51.1	154.2
C		26.5	22.7	62.3	65.0	45.0	27.9	117.5								
E	26	8.0	4.8	49.1	52.2	27.4	44.7	122.1	39.4	12.2	7.8	51.9	55.3	31.9	47.3	123.2
C		37.0	32.4	61.4	71.1	48.5	31.3	90.0								
E	28	22.6	18.1	58.4	70.0	45.4	44.4	102.4	22.6	11.5	6.6	37.8	46.4	19.6	55.7	135.7
C		23.5	19.2	57.6	64.7	41.4	33.8	96.9								
E	31	9.6	4.9	49.8	61.6	34.1	49.5	114.4	46.5	10.9	6.6	45.0	58.1	29.1	52.4	119.1
C		44.0	37.4	62.4	71.4	49.5	30.4	82.5								
E	34	8.4	5.0	46.6	53.9	27.9	45.3	121.1	41.3	10.0	4.6	47.3	50.1	26.3	55.0	127.3
C		27.3	23.3	59.5	78.4	51.8	30.9	118.6								
E	34	9.0	4.5	55.7	58.5	36.2	45.6	140.2	54.7	13.4	7.9	51.8	61.0	35.1	45.9	135.7
C		34.9	30.8	68.5	77.0	58.6	27.5	97.5								

* B.W. = Body weight of rat plus tumor.

Walker tumor 256 by McEwen and Haven (13) and confirmed by others (7, 15).

Rats on elaidin diet.—Table 2 shows the total lipid and fatty acid content, the solid, liquid, and elaidic acid expressed as the percentage of total fatty acids, and the iodine number of the solid and liquid fractions for carcass and tumor and for control rats. With the exception of the rat sacrificed 28 days after transplantation of tumor, the carcass of the tumor-bearing rat contained much less fatty acid than that of the control rat.

Unlike the rats on the Crisco diet (Table 1), the fatty acid composition of the tumor showed no significant qualitative difference from that of the carcass of the host rat, except in the percentage of liquid acids which was higher in tumor than rat (Table 3). On the other hand, the carcasses of the rats with tumors differed markedly in fatty acid composition from those of the controls. The percentage of solid acids was significantly lower with lower iodine numbers and the percentage liquid series, where tumors were small, the fatty acid composition of the carcass of the tumor rat was like the control and different from the tumor; in the elaidin series, where tumors were very large, the carcass of the tumor rat was like the tumor and different from the control rat in fatty acid composition.

DISCUSSION

The progressive growth of Walker carcinoma 256 in rats has previously been shown to cause an increase in total lipid of the blood plasma (3, 10), consisting essentially of an increase in the fatty acid fraction. Likewise, the total lipid content of the host rat was found to decrease significantly (10, 14) during growth of the tumor. That this decrease was primarily in the fatty acid fraction of the total lipid is evident from the results of the present experiments. In spite of the higher fat content of the diet, tumor rats on elaidin suffered a greater depletion of the carcass fatty acids than did rats on Crisco, an effect doubtless due to the

larger tumors. On a diet containing only 8 per cent Crisco, the marked loss of lipid from the carcass of rats during progressive growth of this tumor, as compared to pair-fed rats ingesting exactly the same amount of diet, has been attributed to an increase in the caloric requirement of the host (14).

Although the amount of total (unseparated) fatty acid of tumors was essentially the same on both diets, the greater content of solid, unsaturated acids (elaidic acid) when elaidin was fed

TABLE 3

THE MEAN VALUES AND SIGNIFICANCE OF THE DIFFERENCE BETWEEN THE MEANS FOR THE FATTY ACID COMPOSITION OF TUMOR, OF TUMOR RAT AND OF CONTROL RAT

Summary of Data from Tables 1 and 2

	Tumor		Tumor rat		Control rat
	Mean	P value*	Mean	P value†	Mean
Crisco diet: (Table 1) Tumor per cent B.W.‡ (av. 20.2)					
Solid F.A. per cent	33.9	<0.01	45.3	N.S.§	42.5
Liquid F.A. per cent	66.8	<0.01	55.9	N.S.	58.1
I.N. solid F.A.	29.1	<0.01	48.2	N.S.	47.2
I.N. liquid F.A.	134.7	N.S.	120.5	<0.05 >0.02	106.9
Elaidin diet: (Table 2) Tumor per cent B.W. (av. 37.9)					
Elaidic acid per cent	28.6	N.S.	34.6	<0.01	49.1
Solid F.A. per cent	46.7	N.S.	52.8	<0.01	62.0
Liquid F.A. per cent	51.2	<0.01	45.4	<0.01	30.3
I.N. solid F.A.	54.6	N.S.	59.1	<0.01	71.3
I.N. liquid F.A.	132.5	N.S.	121.5	<0.05 <0.02	100.5

* Significance of the difference between the means of tumor and of carcass of tumor-bearing rats.

† Significance of the difference between the means of tumor-bearing and control rats.

‡ Only data from pairs of rats with tumors over 8 per cent of total body weight are summarized here

§ Not significant.

Summary: In the Crisco series the tumor rat differs from the tumor and is similar to the control rat; in the elaidin series the tumor rat is similar to the tumor and differs from the control rat.

(Table 2) proves that food fat is built into this tumor. The entrance of elaidic acid into the phospholipid fraction of this tumor has been shown previously (9). The increase in solid, unsaturated fatty acids in the tumors caused a decrease in the content of liquid acids without changing the degree of unsaturation of the latter. If elaidic acid replaces oleic acid, as Sinclair believed (19), then the iodine number of the liquid acids should have increased. The reason for the lack of increase in the degree of unsaturation of the liquid acids is not known, but the possibility exists that only so much unsaturation per se is necessary for tumor tissue and growth. If some is available in the solid fatty acid fraction, then the liquid acids, although reduced in amount, need not be more unsaturated.

In the carcass of the rats with tumor, elaidic acid, a solid unsaturated acid with one double bond, made up considerably less of the total fatty acids than in the pair-fed controls that ingested the same amount of this acid. Thus, the tumor rat must be able to use a fatty acid containing one double bond. The significant increase in the degree of unsaturation of the liquid acids in the host rat, as compared to the control, may be attributed to this use by the tumor rat of monoenoic fatty acids. The increase in the iodine number of the liquid fraction is also inversely related to the total amount of fatty acids left in the rat. When roughly two-thirds of the fatty acids were gone (compared to the control), the iodine number rose sharply. A similar relationship was established by Hodge *et al.* (11) for the fasting mouse; the iodine number of the total lipid of the carcass increased slowly until about three-quarters of the utilizable lipid was burned, after which interval the iodine number increased rapidly.

Complete metabolism (burning) of the saturated and only slightly unsaturated acids would spare the highly unsaturated acids for their structural function of incorporation into phospholipids (17). In the host rat, whose fat stores were completely exhausted because of the presence of a large tumor, the fatty acids probably consisted almost entirely of phospholipid fatty acids, which might account for their similarity to the fatty acids of tumor, a tissue rich in phospholipid. Smedley-MacLean (20, 21) ascribed the disappearance of highly unsaturated acids from the skin of rats bearing Walker 256 to their utilization in the formation of tumor tissue. Our data support this theory, since in tumor, regardless of diet, the liquid acids predominated in amount over the solid acids, a situation which might conceivably create a demand for liquid acids on the part of the growing tumor.

The mobilization of fat occurred early in the growth of the tumor (Table 1) while the food intake was normal and before the carbohydrate and protein stores of the animal were exhausted. Both our experiments and those of Smedley-MacLean indicate that fat may be mobilized for the purpose of providing a plentiful supply of the essential unsaturated fatty acids for building tumor tissue. Since the experiments of Hodge (11) and Longenecker (12) show that fat is mobilized nonselectively, the demands of the growing tumor for unsaturated acids for formation of phospholipids and for the esterification of cholesterol (8) might

create an excess of saturated fats to be burned. An excess of nonprotein foods is known to stimulate tissues to increase oxygen consumption, the effect of which would be to force a higher caloric requirement on the host. The marked lipemia also indicates a slow removal of fat from the blood, a condition which would be aggravated by impaired activity of lipase and esterase as established for the tissues of rats bearing tumors other than Walker 256 (4, 6, 22).

SUMMARY

1. Rats bearing Walker carcinoma 256 and rats without tumors that ate the same amount and kind of diet containing either Crisco or elaidin were compared with respect to: (*a*) total lipid content of blood plasma and carcass and (*b*) amount and degree of unsaturation of solid and liquid fatty acids.

2. The total lipid content of the blood plasma was higher, while that of the carcass was lower in rats with tumor. The rise in blood lipids occurred early in tumor growth.

3. In rats with small tumors, the nature of the fatty acids of the carcass differed markedly from that of the tumors and was closely similar to that of the controls.

4. In rats with large tumors, the fatty acids of the carcass were closely similar qualitatively to those of the tumor and significantly different from those of the pair-fed controls.

5. The degree of unsaturation of the liquid fatty acids was greater in carcasses of tumor-bearing rats than in controls.

6. In the carcass of the tumor-bearing rat the degree of unsaturation of the liquid fatty acids was inversely related to the fatty acid content of the rat.

7. In brief, the effect of the growing tumor on the host seems to be (*a*) to cause a lipemia, (*b*) to decrease the fatty acid content of the host, (*c*) to cause the ratio of solid to liquid fatty acids to become like that in tumor, and (*d*) to increase the degree of unsaturation of the liquid acids of the host.

REFERENCES

1. BLOOR, W. R. The Determination of Small Amounts of Lipid in Blood Plasma. J. Biol. Chem., **77**:53–73, 1928.
2. ———. A Colorimetric Procedure for the Determination of Small Amounts of Fatty Acid. J. Biol. Chem., **170**:671–74, 1947.
3. BLOOR, W. R.; HAVEN, F. L.; and ASHWORTH, E. Lipids of the Adrenals and Blood Plasma in Cancer. Cancer Research, **10**:205, 1950.
4. EDLBACHER, S., and NEBER, M. Über das Lipasespaltungsvermögen der Säugeorgane im gesunden und tumorkranken Organismus. Ztschr. f. physiol. Chem., **233**:265–75, 1935.
5. GOULDEN, C. H. Methods of Statistical Analysis, Chapter IV. New York: John Wiley & Sons, Inc., 1939.
6. GREEN, H. N., and JENKINSON, C. N. Changes in the Esterase and Fat Content of the Serum Induced by Cancer and Cancer-producing Agents. Brit. J. Exper. Path., **15**: 1–14, 1934.
7. GREEN, J. W., JR.; BENDITT, E. P.; and HUMPHREYS, E. M. The Effect of Protein Depletion on the Host Response to Transplantable Rat Tumor Walker 256. Cancer Research, **10**:769–74, 1950.
8. HAVEN, F. L. Tumor Lipids. The Comparative Lipid Content of the Periphery and Center. Am. J. Cancer, **29**: 57–62, 1937.
9. ———. The Phospholipid Metabolism of Tumors. J. Biol. Chem., **118**:111–21, 1937.
10. HAVEN, F. L.; BLOOR, W. R.; and RANDALL, C. Lipids of the Carcass, Blood Plasma, and Adrenals of the Rat in Cancer. Cancer Research, **9**:511–14, 1949.
11. HODGE, H. C.; MACLACHLAN, P. L.; BLOOR, W. R.; STONEBURG, C. A.; OLESON, M. C.; and WHITEHEAD, R. Lipids of the Fasting Mouse. I. The Relation between Carcass Lipids and Liver Lipids. J. Biol. Chem., **139**: 897–916, 1941.
12. LONGENECKER, H. E. Deposition and Utilization of Fatty Acids. I. Fat Synthesis from High Carbohydrate and High Protein Diets in Fasted Rats. J. Biol. Chem., **128**:645–58, 1939.
13. MCEWEN, H. D., and HAVEN, F. L. The Effect of Carcinosarcoma 256 on the Water Content of the Liver. Cancer Research, **1**:148–50, 1941.
14. MIDER, G. B.; SHERMAN, C. D., JR.; and MORTON, J. J. The Effect of Walker Carcinoma 256 on the Total Lipid Content of Rats. Cancer Research, **9**:222–24, 1949.
15. SHERMAN, C. D., JR.; MORTON, J. J.; and MIDER, G. B. Potential Sources of Tumor Nitrogen. Cancer Research, **10**:374–78, 1950.
16. SINCLAIR, R. G. The Metabolism of the Phospholipids. III. The Comparative Influence of Various Fats on the Degree of Unsaturation of the Phospholipids and Neutral Fat in the Tissues of the Rat. J. Biol. Chem., **92**:245–56, 1931.
17. ———. The Metabolism of the Phospholipids. VII. Further Evidence of the Selection and Retention of Unsaturated Fatty Acids by Phospholipids of Animal Tissues. *Ibid.*, **111**:275–84, 1935.
18. ———. The Metabolism of the Phospholipids. VI. Relative Proportions of Saturated and Unsaturated Fatty Acids in Phospholipids of Different Degrees of Unsaturation. *Ibid.*, pp. 261–73.
19. ———. The Metabolism of the Phospholipids. VIII. The Passage of Elaidic Acid into Tissue Phospholipids. Evidence of the Intermediary Rôle of Liver Phospholipid in Fat Metabolism. *Ibid.*, pp. 515–26.
20. SMEDLEY-MACLEAN, I., and NUNN, L. C. A. Fat-deficiency Disease of Rats. The Relation of the Essential Unsaturated Acids to Tumour Formation in the Albino Rat on Normal Diet. Biochem. J., **35**:983–89, 1941.
21. SMEDLEY-MACLEAN, I., and HUME, E. M. Fat-deficiency Disease of Rats. The Influence of Tumour Growth on the Storage of Fat and of Polyunsaturated Acids in the Fat-starved Rat. Biochem. J., **35**:996–1002.
22. TROESCHER, E. E., and NORRIS, E. R. A Micro Blood Esterase Determination Applied to Studies of Rats Bearing Adenocarcinoma. J. Biol. Chem., **132**:553–57, 1940.
23. YASUDA, M. The Determination of the Iodine Number of Lipids. J. Biol. Chem., **94**:401–9, 1931.

Effects of Anterior Hypophyseal Transplants on Intrasplenic Ovarian Grafts*

MARTIN SILBERBERG, RUTH SILBERBERG, AND HELEN V. LEIDLER

(*Snodgras Laboratory, Hospital Division, City of St. Louis, Mo.*)

In virgin mice of strain A, anterior hypophyseal transplants increased the incidence of mammary cancers; in castrate males bearing hypophyseal and ovarian grafts, the number of mammary cancers exceeded that noted after transplantation of ovaries alone. These findings were interpreted as owing to the release of gonadotrophins by the hypophyseal transplants, which stimulate the output of estrogen by the animal's own or the grafted ovaries (6, 9, 10). Therefore, it seemed of interest to investigate whether or not hypophyseal transplants might exert effects on intrasplenic ovarian grafts which are already exposed to increased activity of the animal's own hypophysis.

MATERIALS AND METHODS

One hundred and twenty-three mice of the closely inbred strain A were ovariectomized at the age of 3–4 weeks. At the time of ovariectomy, one ovary was transplanted into the spleen. Sixty-two of the animals received no further treatment. One to 3 weeks after ovariectomy, the remaining 61 mice received grafts of four anterior hypophyses obtained from closely related donors 1–3 months of age. Fifty-one mice received anterior hypophyses from male donors, seven mice received hypophyses from female donors, and two mice received combinations of two male and two female, or one male and three female hypophyses, respectively. In one case, the sex of the donors was not recorded. The technic of transplantation has been described previously (7, 9). The mice were kept on a stock diet of Purina Laboratory Chow and water *ad libitum*. Groups of animals were sacrificed 1, 2, 4, 5, 6, 9, or 12 months following ovariectomy. At necropsy, hypophyseal transplants or fragments of tissue suggestive of such transplants, spleen with ovarian grafts, four mammary glands, one leg, and some adrenals were removed, fixed in Bouin's solution, and imbedded in paraffin. Semi-serial sections stained with hematoxylin and eosin were prepared for microscopic studies.

* This investigation was supported by a research grant from the National Cancer Institute, Public Health Service, and a grant from the Brant Fund of the American Medical Association.

Received for publication March 19, 1951.

OBSERVATIONS

Of 123 splenic transplants originally made, 66 were suitable for the purpose of the present investigation. The remainder had to be discarded because of fibrous adhesions around the spleen or because of the failure of the grafts to take or to stay in the spleen. Altogether, 30 ovarian transplants of mice carrying no hypophyseal grafts and 36 ovarian transplants of mice carrying hypophyseal grafts were studied. Of the latter mice, 31 had received male hypophyses, and 4 animals had received female hypophyses, the remaining mouse being the one recipient for whom the sex of the donors was not recorded. Details of the experiments are shown in Tables 1 and 2.

The fate of subcutaneous hypophyseal grafts has been described previously (7, 9, 10), and functioning grafts have been illustrated by us (9, 10). Transplanted hypophyses may remain alive and function through life; yet, with an increasing interval of time after grafting, many transplants undergo fibrosis or resorption. In 16 of the 36 animals used for the present investigation, hypophyses or remnants thereof could be identified microscopically. The results of cytological and cytochemical studies of such grafts, which are presently being carried out, will be reported at a later date.

MICROSCOPIC EXAMINATION

In Tables 1 and 2, the interval from the time of grafting of the ovaries, the number of mice, the number of ovarian tumors and of hyperplastic ovaries found, and the microscopic findings are given for all animals. The grade of the change is indicated by one or several + signs placed in parentheses behind the number of mice showing the the respective change. In Table 3, the tumors are classified according to their microscopic structure. Uniformly enlarged and proliferating ovaries, yet containing the typical ovarian structures, were

TABLE 1

Ovarian Grafts Not Grossly Enlarged

Months after grafting of ovaries	Without hypophyseal grafts (7 mice) No. of ovaries showing: No. of mice	Stimulation of follicles	Presence of tubules	Increase of: Young lutein tissue	Old lutein tissue	With hypophyseal grafts (11 mice) No. of ovaries showing: No. of mice	Stimulation of follicles	Presence of tubules	Increase of: Young lutein tissue	Old lutein tissue
1-5	3	3 (+)	0	3 (+)	1 (+)	5	2 (+) 3 (++)	3 (+) 1 (++)	5 (+)	1 (+) 1 (++)
6-8	2	2 (+)	0	2 (+)	1 (+)	1	1 (+)	1 (+)	1 (+)	1 (++)
9-12	2	2 (+)	1 (+)	1 (+) 1 (++)	1 (+) 1 (++)	5*	1 (+) 3 (++)	2 (++)	2 (+) 1 (++)	1 (++) 3 (+++)
Irrespective of duration of exp.	7	7 (+)	1 (+)	6 (+) 1 (++)	3 (+) 1 (++)	11	4 (+) 6 (++)	4 (+) 3 (++)	8 (+) 1 (++)	1 (+) 3 (++) 3 (+++)

* One animal had an early mammary tumor.

TABLE 2

Ovarian Grafts Grossly Enlarged

Mos. after grafting of ovaries	Without hypophyseal grafts (23 mice) No. of hyperplastic ovaries showing: No. of mice	No. of ovarian tumors	No. of hyperplastic ovaries	Stimulation of follicles	Presence of tubules	Increase of: Young lutein tissue	Old lutein tissue	With hypophyseal grafts (25 mice) No. of hyperplastic ovaries showing: No. of mice	No. of ovarian tumors	No. of hyperplastic ovaries	Stimulation of follicles	Presence of tubules	Increase of: Young lutein tissue	Old lutein tissue
1-5	7	3*	4	3 (+) 1 (++)	1 (+)	4 (+)	1 (+)	6	5*	1	1 (++)	1 (+)	1 (++)	1 (++)
6-8	8	4*	4	1 (+) 3 (++)	1 (+)	3 (+) 1 (++)	1 (+) 1 (++)	7	4*	3	2 (+) 1 (++)	2 (+) 1 (++)	2 (+++)	2 (+++)
9-12	8	6*	2	1 (+) 1 (++)	1 (++)	2 (+)	1 (+)	12	9*†	3	3 (+)	2 (+++)	3 (+++)	1 (++) 2 (+++)
Irrespective of duration of exp.	23	13	10	5 (+) 5 (++)	2 (+) 1 (++)	9 (+) 1 (++)	3 (+) 1 (++)	25	18*	7	5 (+) 2 (++)	3 (+) 1 (++) 2 (+++)	1 (++) 5 (+++)	2 (++) 4 (+++)

* Classification is given in Table 3.

† One animal had, in addition, a mammary tumor.

TABLE 3

Microscopic Classification of the Tumors

Months after ovarian grafting	Without hypophyseal grafts (30 mice) Total no. of tumors and cysts	Types of growth	With hypophyseal grafts (36 mice) Total no. of tumors and cysts	Types of growth
4–5	3	3 granulosa-cell tumors (2 cystic)	5	3 granulosa-cell tumors (1 cystic) 1 mixed tumor 1 luteoma
6–8	4	4 granulosa-cell tumors (2 cystic)	4	1 granulosa-cell tumor (cystic) 1 mixed tumor 1 luteoma 1 large cyst with lutein tissue*
9–12	6	3 granulosa-cell tumors (2 cystic) 2 mixed tumors (1 cystic) 1 luteoma	9	3 mixed tumors 3 luteomas† 2 large cysts with lutein tissue‡ 1 large cyst§
Irrespective of duration of exp.	13	10 granulosa-cell tumors (6 cystic) 2 mixed tumors 1 luteoma	18	4 granulosa-cell tumors 5 mixed tumors 5 luteomas 4 large cysts (3 with lutein tissue)

* Measurements: 8×8×8 mm.

† One animal had, in addition, a mammary tumor.

‡ Measurements: 5×5×5 mm.; 4×4×5 mm.

§ Measurements: 6×7×8 mm.

considered as hyperplastic; tumors were diagnosed when proliferating nodules free from pre-existing ovarian tissue were seen. The following description and symbols apply to the findings in the grossly nonenlarged as well as hyperplastic ovaries.

Follicles.—(+) stimulation: numerous mitotically proliferating primordial and small or medium-sized follicles are seen; (+ +) stimulation: many large proliferating follicles are present with or without follicular fluid.

Tubules.—(+): some tubules lined by cuboidal epithelium are found—they are often situated near the surface or the hilum of the ovary; (+ +): several foci of tubular glands are noted; (+ + +): small adenomatous formations are observed.

Young lutein tissue.—An increase of (+) means the presence of large foci of eosinophilic lutein cells; of (+ +) that of coalescent layers of lutein tissue; of (+ + +) that of large lutein bodies occupying most of the ovary.

Old lutein tissue.—consists of large, vacuolated, clear cells or of cells containing fine, yellowish, granular "ceroid" pigment. An increase of (+) is characterized by small isolated groups of cells, one of (+ +) by coalescent foci of old lutein tissue, and one of (+ + +) by diffuse replacement of the ovarian tissue by ceroid cells.

Intrasplenic Ovarian Grafts without Hypophyseal Transplants

Grossly nonenlarged ovarian grafts (7 of 30 mice).—Proliferating follicles were present in all grafts removed 1–12 months after transplantation. A few tubules were noted in a single ovary out of seven removed 12 months after grafting. Increased amounts of young lutein tissue were seen in all ovaries of the younger age groups, whereas old lutein tissue was found only in one of three grafts 5 months old, and in one of two grafts 7 months old. Only after 9–12 months of intrasplenic growth did the ovaries contain abundant old lutein tissues.

Grossly enlarged ovarian grafts (23 of 30 mice).—One to 5 months after transplantation, focal hyperplasia was noted in four, and granulosa-cell tumors in three of seven mice, 5 or 6 months old. Two of the tumors showed cysts, and one disclosed tubules and teleangiectases. Six to 8 months after transplantation, granulosa-cell tumors were present in four of eight grafted ovaries. Two of these growths contained blood, phagocytosed blood pigment or small cysts, and some tubules. The longer the transplant was allowed to grow, the more conspicuous the latter elements as well as old lutein tissue became. After 9–12 months, tumors were found in six of eight ovarian grafts: three were of granulosa-cell type, two were mixed tumors, and one was a luteoma. In all hyperplastic ovaries, follicular growth was stimulated, and in half it was marked. Tubules were observed in one of four grafts each 5 or 8 months old, and they were conspicuous in one of two grafts 10 months of age. A great deal of young lutein tissue was present in the ovaries of all age groups. One of four grafts 5 months old, and three of six grafts 6–12 months of age showed old lutein tissue.

Intrasplenic Ovarian Grafts with Subcutaneous Hypophyseal Transplants

Grossly nonenlarged ovarian grafts (11 of 36 mice).—One to 5 months after transplantation, four of five animals showed living hypophyseal grafts. Three mice had received male and one had received female hypophyses, the fifth being the one in which the sex of the donors had not been recorded. Follicular growth was slightly stimulated in two, and markedly intensified in three of five ovarian grafts; in four ovaries tubules were present. All ovarian transplants contained increased numbers of young lutein cells; in one graft, there were some, and in another numerous foci of old lutein cells. One ovarian graft was fibrosed in spite of the presence of living hypophyseal tissue (male donors). The one mouse examined 7 months after the transplantation of male hypophyses showed, in the ovarian graft, stimulation of follicular and tubular growth and large amounts of young and old lutein tissue. Hypophyseal grafts were not identified microscopically in this case. Nine to 12 months after transplantation, follicular stimulation was accentuated in three, moderate in one, and absent in another of five ovarian grafts. Two ovaries contained clusters of tubules. Increased layers of young lutein tissue were noted in three grafts, and in four of five ovaries large areas were replaced by old lutein tissue. Hypophyseal grafts were not found except for one mouse in which a small lutein cyst had developed. In all experiments of this group, male hypophyses had been grafted.

Grossly enlarged ovarian grafts (25 of 36 mice).—One to 5 months after transplantation, hyperplastic granulosa cells were seen in one ovary in association with viable hypophyseal tissue (male donors). There were tubules and abundant young and old lutein tissue in this ovary. Mitoses of young lutein cells were particularly impressive in areas directly underneath the surface epithelium of the ovary. Tumors were found in five of six mice 5 or 6 months old. Three of the neoplasms were granulosa-cell tumors (male hypophyses had been transplanted in two, and female hypophyses in one mouse), one (Fig. 1) was a mixed tumor (female hypophyses had been grafted), and one was a lu-

teoma (female hypophyses had been grafted). Hypophyseal grafts were identified microscopically in four tumor-bearing mice. In one animal with a granulosa-cell tumor, no hypophyseal grafts were found. Small cysts, hemorrhages, phagocytosis of blood pigment, and thin-walled dilated vessels were observed frequently. Six to 8 months after transplantation of hypophyses (male hypophyses had been grafted into all recipients), four ovarian tumors were found, while three transplants were hyperplastic. In the latter, follicular growth was stimulated; tubular growth was present in three grafts, and it had reached major proportions in one of them; large amounts of fresh and old lutein tissue were noted in two ovaries. Of the tumors, one was a granulosa-cell tumor, one a mixed tumor (Fig. 2), one a luteoma (Fig. 3), and one was a large cyst with lutein tissue at the periphery. In the mouse bearing a granulosa-cell tumor, viable hypophyseal tissue was seen, while in the three remaining animals the hypophyseal grafts had undergone fibrosis. All the mice sacrificed 9–12 months after transplantation of the hypophyses had received pituitaries from male donors. Hyperplastic ovaries were found in three and tumors in nine grafted ovaries. These grafts disclosed follicular stimulation, and two of them showed adenoma-like formations of tubules and focal fibrosis. Abundant young and old lutein tissue was seen in three grafts. Of the tumors, three were mixed tumors, three were luteomas, and three were large cystic growths, two of them containing lutein tissue at the periphery. Viable hypophyseal tissue was found with one luteoma and with one large cyst containing lutein tissue at the periphery.

DISCUSSION

The present findings regarding tumor growth in intrasplenic ovarian grafts are essentially in agreement with those of other investigators (1–5). Moreover, we made some additional observations by studying the changes in non-neoplastic or preneoplastic grafts. There occurred in these transplants changes similar to those found in aged ovaries of untreated mice, namely, formation of epithelial tubules, cysts, and clear or ceroid-laden lutein cells. In mice of strain A, these findings become common between the ages of 14 and 16 months but are rare in ovaries less than 1 year old (6). In intrasplenic ovarian grafts of mice receiving no further treatment, these age changes began to appear as early as 5 months after transplantation, i.e., in ovaries about 6 months old. In 8-month-old ovaries, grafts left in the spleen for 7 months, these changes were seen regularly. Presumably, the same factors that are involved in the pathogenesis of tumors are also responsible for the acceleration of age changes in the nontumorous intrasplenic ovarian grafts.

Up to 8 months following transplantation, hypophyseal transplants were identified in eight of nine mice with intrasplenic tumors. Nine to 12 months following transplantation, only two of nine tumor-bearing mice showed hypophyseal grafts. These findings are in harmony with the previously reported progressive fibrosis and resorption of such transplants (7, 9, 10). In the absence of living grafts it is obviously impossible to state whether or not the grafts had originally taken and functioned. There is, however, indirect evidence in support of these possibilities, such as the microscopic appearance of the mammary glands. Nine to 12 months after transplantation, all mice with intrasplenic tumors showed impressive stimulation of the mammary glands, ranging in degree from ductal proliferation and secretion to acinar growth. Estrogen can be ruled out as the cause of this stimulation, since intrasplenic tumors would not grow in the presence of estrogen. On the other hand, pituitary secretions, as produced by grafted hypophyses, have been shown to enhance mammary growth (7, 9, 10).

In spite of the comparatively limited number of experiments, the following conclusions may be made:

Under the influence of subcutaneous anterior hypophyseal grafts, the intrasplenic ovarian transplants underwent changes that distinguished them from similar transplants not exposed to equally excessive hypophyseal stimulation. The differences between these two types of ovarian grafts were quantitative or concerned the time of appearance of age changes within the grafts. The processes of growth and aging were hastened in intrasplenic ovarian grafts of mice that had received hypophyseal transplants: follicular growth and development were stimulated; proliferation of theca cells was accentuated, and the latter cells were, at an early date, converted into lutein cells. An early increase of lutein tissue ensued, and, within the latter, old lutein tissue became rapidly predominant over young lutein cells. Whether or not androgenic hormone is produced by the clear cells (8) has no bearing on the present investigation. The increased growth of lutein cells is probably attributable to the action of the luteinizing hormone produced by the transplanted hypophyses, which in 31 out of 36 recipients were derived from male donors. Moreover, under the influence of anterior hypophyseal transplants, epithelial tubules proliferated and appeared prematurely. These findings may likewise be interpreted as an acceleration

of age changes within the grafted ovaries caused by the transplanted hypophyses. The present experiments were terminated after 1 year of observation. This may represent too early a stage to determine whether or not all nontumorous intrasplenic ovaries will ultimately undergo cyst formation—possibly a manifestation of exhaustion after excessive growth—as do untreated senile ovaries. The increased number and size of cysts appearing in grafts toward the end of the first year of observation certainly suggest such a possibility.

Regarding the intrasplenic ovarian tumors, there was no evidence that the ultimate amount of newly formed neoplastic tissue was larger in the presence than in the absence of additional hypophyses. There was, however, in mice carrying hypophyseal grafts a trend toward an accelerated rate of tumor growth during the early stages following transplantation. In correspondence with our findings in nontumorous grafts, the neoplasms themselves showed, under the influence of hypophyseal transplants, an increased occurrence of lutein tissue—an effect that has recently been observed following injections of pregnant mare serum into mice bearing intrasplenic ovaries (4). In the present experiments, a mixed tumor and a luteoma were seen as early as 5 months after transplantation; and after 9–12 months, three mixed tumors, three luteomas, and two cysts with lutein tissue were found. This was in marked contrast to the findings in intrasplenic ovarian tumors of mice not receiving hypophyseal grafts in which luteomas are rare (4, 5), and in which we observed only one as late as 9 months after transplantation. Conversely, granulosa-cell tumors were noted in mice of all age groups not receiving hypophyseal grafts, whereas in the presence of the latter we found no granulosa-cell tumors in old ovarian grafts.

SUMMARY

In ovariectomized mice of strain A, the effects of subcutaneous anterior hypophyseal transplants on intrasplenic ovarian grafts were studied. The hypophyseal transplants promoted growth and age changes in the grafted ovaries: proliferation of follicles and lutein tissue was stimulated; epithelial tubules and old lutein tissue appeared prematurely and were increased in amount; intrasplenic tumors tended to appear early; and the ratio of granulosa-cell tumors to luteomas shifted in favor of the latter. These effects are considered to be caused by the action of both the follicle-stimulating and luteinizing hormones given off by the grafted hypophyses.

REFERENCES

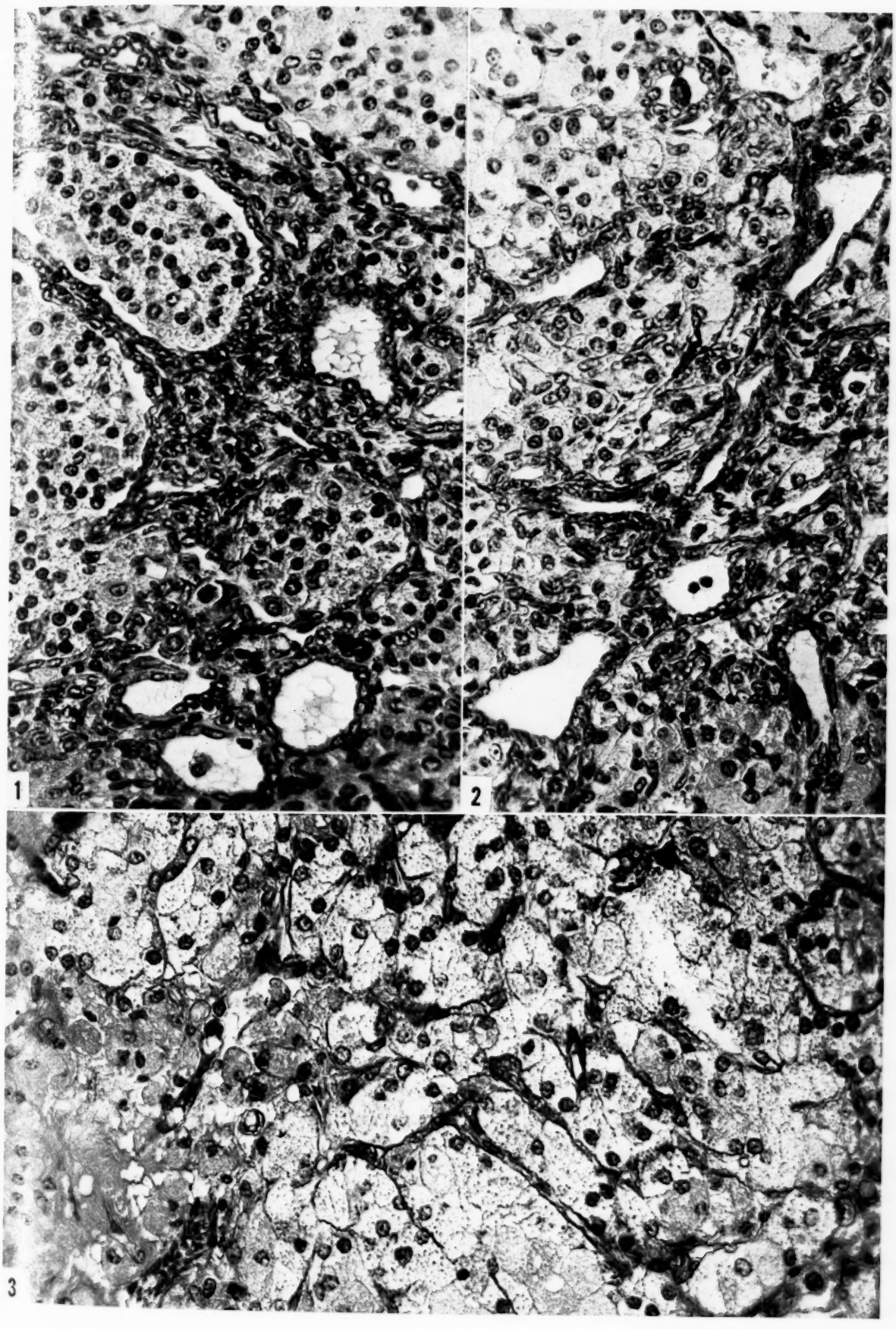
1. Biskind, M. S., and Biskind, G. R. Development of Tumors in Rat Ovary after Transplantation into the Spleen. Proc. Soc. Exper. Biol. & Med., **55**:176–79, 1944.
2. Furth, J., and Boon, M. C. Induction of Ovarian Tumors in Mice by X-Rays. Cancer Research, **7**:241–45, 1947.
3. Furth, J., and Sobel, H. Neoplastic Transformation of Granulosa Cells in Grafts of Normal Ovaries into Spleens of Gonadectomized Mice. J. Nat. Cancer Inst., **8**:7–16, 1947.
4. Gardner, W. U. Hormonal Imbalance in Tumorigenesis. Cancer Research, **8**:397–411, 1948.
5. Li, M. H., and Gardner, W. U. Further Studies on the Pathogenesis of Ovarian Tumors in Mice. Cancer Research, **9**:35–41, 1949.
6. Loeb, L. Aging Processes in the Ovaries of Mice Belonging to Strains Differing in the Incidence of Mammary Cancer. Arch. Path., **46**:401–40, 1948.
7. Loeb, L., and Moskop Kirtz, M. The Effects of Transplants of Anterior Lobes of the Hypophysis on the Growth of the Mammary Gland and on the Development of Mammary Gland Carcinoma in Various Strains of Mice. Am. J. Cancer, **36**:56–82, 1939.
8. Pfeiffer, C. A., and Hooker, C. W. Early and Late Effects of Daily Treatment with Pregnant Mare Serum on the Ovaries of Mice of the A Strain. Anat. Rec., **84**:311–29, 1942.
9. Silberberg, M., and Silberberg, R. Mammary Growth in Orchidectomized Mice Grafted with Anterior Lobes of Hypophyses and Ovaries at Various Ages. Arch. Path., **49**:733–51, 1950.
10. Silberberg, R., and Silberberg, M. Mammary Cancer in Castrate Male Mice Receiving Ovarian and Hypophyseal Grafts at Different Ages. Proc. Soc. Exper. Biol. & Med., **70**:510–13, 1949.

Figs. 1–3.—Sections through intrasplenic ovarian grafts of ovariectomized mice of strain A having received four anterior hypophyses at 4–6 weeks of age.

Fig. 1.—Section through a mixed tumor 3 months after transplantation of four female hypophyses. Viable hypophyses were identified. There is formation of epithelial tubules and lutein bodies. Mag. ×400.

Fig. 2.—Section through a mixed tumor 6 months after transplantation of four male hypophyses. Fibrosed hypophyseal tissue was found microscopically. Epithelial tubules and lutein tissue are seen. Mag. ×400.

Fig. 3.—Section through a luteoma 7 months after transplantation of four male hypophyses. Viable hypophyses were identified. The tumor consists of large lutein cells. Mag. ×400.


1
2
3

Adrenal Cortical Function and Radiation-induced Lymphoid Tumors of Mice*

HENRY S. KAPLAN, SUMNER N. MARDER,† AND MARY B. BROWN

(Department of Radiology, Stanford University School of Medicine, San Francisco, Calif.)

The acute involutional response of lymphoid tissues to the administration of adrenal cortical extract and the lymphoid hyperplasia which occurs after bilateral adrenalectomy are now well established (4, 16). These physiological responses suggest that the level of adrenal cortical function might influence the development of lymphoid tumors in mice exposed to total body roentgen irradiation. These tumors are malignant lymphocytic or lymphoblastic lymphomas, which have been described in detail elsewhere (6, 7). The present report is concerned with a series of four experiments designed to study the role of adrenal cortical function in the development of these neoplasms.

METHODS

Strain C57 black mice, of both sexes, were used. The observed spontaneous incidence of lymphomas in this strain varies from 0 to 8 per cent, with virtually all tumors appearing after 1 year of age (13). In contrast, the incidence of lymphoid tumors within 1 year after whole-body irradiation has approximated 60–90 per cent.

Except for the adrenalectomized animals of Experiment 4, all mice were maintained under identical laboratory conditions on Purina Laboratory Chow and water ad libitum. Animals that died soon after irradiation or became too autolyzed for autopsy were discarded. All other animals were examined at autopsy; representative tissues were sectioned, stained with hematoxylin and eosin, and studied microscopically.

In Exp. 1, littermate animals were distributed among four groups, all of which received total body irradiation[1] starting at 6 weeks of age. The initial dose of 150 r/air on 5 consecutive days was reduced to 100 r/air/day×5, because of excessive mortality. One group received no further treatment; the others were injected with adrenal cortical extract[2] starting at 4, 6, and 12 weeks of age, respectively. In Exp. 2, littermate animals 4–6 weeks of age were assigned to two groups, both of which received total body irradiation.[1] Five consecutive daily doses of 100 r/air each were given. One group, on the first day of irradiation, also received a pellet of desoxycorticosterone acetate by trocar implantation.[3]

The more complex design of Exp. 3 was based upon earlier evidence that radiation-induced lymphoid tumors of strain C57 black mice tend to originate in the thymus (8) and that the pathogenesis of these tumors involves an indirect mechanism as well as a possible direct effect of the ionizing radiation (9). Local or total body irradiation and injections of cortisone acetate were given in various combinations as indicated in Table 3.

The fourth experiment was designed to test the effect of bilateral total adrenalectomy on mortality and lymphoma incidence after roentgen irradiation of mice. Earlier studies in this laboratory have established an operative technic for adrenalectomy of mice, which is followed by a sustained reduction in adrenal cortical secretion, as measured by a potassium stress test (14). It has been found that a simple dietary regimen of bread and milk, supplemented during the immediate post-operative period by adrenal cortical extract, is adequate to sustain normal growth and development in mice adrenalectomized by this technic.

* These investigations were initiated in the Section of Biophysics, National Cancer Institute, Bethesda, and continued in the Department of Radiology, Stanford University School of Medicine, with the aid of a grant from the National Cancer Institute, Public Health Service.

† Former National Cancer Institute research fellow and trainee in cancer. Present address: Mallinckrodt Institute of Radiology, Washington University School of Medicine, St. Louis, Mo.

Received for publication April 14, 1951.

[1] Physical factors: 186 kvP, 20 ma., 0.25 mm. Cu and 0.55 mm. Al added filter, focus skin distance 47 cm., output 66 r/min.

[2] Lipo-adrenal extract in oil (Upjohn), 0.1 cc. intramuscularly 3 times per week for 2 weeks.

[3] Pellets containing 10–15 mg. of desoxycorticosterone acetate (Percorten) were supplied through the courtesy of Dr. Ernst Oppenheimer, Ciba Pharmaceutical Products, Summit, N.J. These were stated to have an average absoprtion rate of approximately 0.1–0.2 mg/day.

In the present experiment, littermate animals were distributed among three groups, the first two of which were subjected to adrenalectomy and the third to sham-operation at 1 month of age. The first group received no further treatment; the sham-operated and the other adrenalectomized group received total body irradiation in four doses of 175 r/air at 2-week intervals starting 6–10 days after operation. The adrenalectomized groups received two 0.1-cc. intramuscular injections of adrenal cortical extract[2] immediately after operation and 4 days later. All groups were placed on a diet of bread soaked in milk enriched with 2 per cent Brewer's yeast. Because of the initially high mortality in the adrenalectomized-irradiated group, all adrenalectomized mice were soon placed on 0.9 per cent sodium chloride and 5 per cent glucose solution as drinking water, in addition.

RESULTS

The results of these four experiments are summarized in Tables 1, 2, 3, and 4, respectively. Adrenal cortical extract, in the dose given, had no significant effect on radiation-induced lymphoid tumor incidence (Table 1). The administration of desoxycorticosterone acetate was also without effect (Table 2).

In Exp. 3, no lymphomas developed after cortisone administration alone. However, injections of cortisone started either concurrently with whole-body irradiation or 6 weeks thereafter effected a distinct inhibition of lymphoma development (Table 3).[4] Cumulative incidence curves (Chart 1) appear to be reaching plateau levels (10). The resistance of mice to the development of lymphomas after *local* irradiation (9) was not modified by cortisone administration.

In Exp. 4, adrenalectomized animals subjected

TABLE 1

EFFECT OF ADRENAL CORTICAL EXTRACT ON RADIATION-INDUCED LYMPHOID TUMOR INCIDENCE

Group	Age at x-ray* (weeks)	Age at start of adrenal extract† (weeks)	No. of mice	Lymphoid tumors No.	Lymphoid tumors Per cent	Av. induction time (days)
I	6	....	35	15	37	178
II	6	4	32	8	25	205
III	6	6	51	9	29	200
IV	6	12	33	15	45	193

* Five doses of 100 r each.

† Lipo-adrenal Cortex Extract in oil (Upjohn); 0.1 cc. intramuscularly 3 times a week for 2 weeks.

TABLE 2

LACK OF EFFECT OF DESOXYCORTICOSTERONE ACETATE PELLETS ON LYMPHOMA INDUCTION BY X-RAYS

Group	Treatment	No. of mice	Lymphoid tumors No.	Lymphoid tumors Per cent	Av. induction time (days)
I	X-ray*	35	10	29	250
II	X-ray* plus Doca†	35	9	26	216

* Five daily doses of 100 r each.

† Doca pellets, 10–15 mg. (Percorten, Ciba) Av. absorption rate, 0.1–0.2 mg/day } at 4–6 weeks of age.

[4] The data of groups II and III and of groups II and IV have been treated as separate 2×2 contingency tables in tests of significance by the χ^2 test, using Yates' correction for continuity (from R. A. Fisher and F. Yates, *Statistical Tables for Biological, Agricultural, and Medical Research* [3d ed.; Edinburgh and London: Oliver and Boyd, 1949], pp. 4–5). Comparison of groups II and III yielded a value for χ^2, with 1 degree of freedom, of 19.38, indicating a highly significant difference ($P = <0.001$). The value of χ^2 for groups II and IV was 7.88 ($P = <0.01, >0.001$), which is also significant. Similar computations for groups II and III of Exp. 4 yield a χ^2 of 12.9, with a probability of less than 0.001.

TABLE 3

EFFECT OF CORTISONE ON DEVELOPMENT OF RADIATION-INDUCED LYMPHOMAS IN STRAIN C57 BLACK MICE

Group	X-ray treatment* Dose	X-ray treatment* Region	Cortisone†	Determinate no. of mice	Mice with lymphoma No.	Mice with lymphoma Per cent	Av. latent period (days)	No. mice dead (neg.)	No. mice alive
I	none		yes	39					39
II	150 r × 4 every 4 days (600 r)	Whole body	no	50	48	96	147		2
III	same	" "	yes	48	27	56	168	1	20
IV	same	" "	yes, 6 weeks post-x-ray	23	16	70	174		7
V	300 r × 4 every 4 days (1,200 r)	Thymus	yes	46				1	45
VI	150 r × 4 every 4 days (600 r)	Shielded	yes	46	1	2	118		45
VII	same	Shielded	no	54					54

* Physical factors: 120 kvP, 9 ma, 0.25 mm. Cu plus 1.0 mm. Al added filter, HVL 0.36 mm. Cu, output 32.2 r/min., target to mid-mouse distance 30 cm. Treatment started at 33 ± 3 days of age.

† Cortone acetate (Merck), 5 mg/cc, 0.05 cc (0.25 mg.) subcutaneously, 2 injections per week for 10 weeks, starting at time of first x-ray treatment, except in Group IV.

to a dose of total body irradiation which yielded a negligible number of deaths in sham-operated controls suffered a very striking increase in mortality (Chart 2). The incidence of lymphoid tumors observed in surviving adrenalectomized, irradiated mice was significantly[4] higher than that in sham-operated, irradiated controls (Table 4, Chart 3). This is consistent with an earlier report (12) that adrenalectomy accelerates the appearance of spontaneous leukemias in strain C58 mice.

DISCUSSION

It appears that cortisone inhibits and adrenalectomy enhances the susceptibility of mice to the development of lymphoid tumors after total body irradiation. These observations, together with experimental evidence (9, 11) that the genesis of these tumors depends in part upon an indirect, presumably systemic mechanism, suggest strongly that adrenal cortical responses may be involved in the induction process.

If the indirect effect of irradiation were simply to stimulate the release of adrenal cortical hormone, it might have been expected that exogenously supplied hormone-like material (cortisone) would act synergistically with local mediastinal irradiation to yield thymic lymphomas. Conversely, if the direct effect of irradiation upon the thymus were simply a nonspecific involution, the involutional stimulus provided by cortisone might have been expected to substitute for it. Neither response was observed (Groups V and VI, Table 3); instead, the action of adrenal corticoids proved to be inhibitory.

TABLE 4

EFFECT OF ADRENALECTOMY ON MORTALITY AND INCIDENCE OF RADIATION-INDUCED LYMPHOMAS IN STRAIN C57 BLACK MICE

Group	Operation*	X-ray†	Initial no. of mice	Mortality No.	Mortality per cent	No. mice surviving 3 mo. post x-ray	Mice with lymphoma No.	Mice with lymphoma Per cent	Av. latent period	No. mice dead	No. mice alive
I	Adrenalectomy		30	6	20	24	0			2	22
II	"	175 r × 4 every 2 weeks (700 r)	60	40	67	20	19	95	195	1	0
III	Sham	same	61	2	3	59	27	46	204		32

* All operations were done when mice were 33 ± 3 days of age.

† X-ray treatment was started 6–10 days post-operatively. Physical factors: 120 kvP, 9 ma., 0.25 mm. Cu 1.0 mm. Al added filter, HVL 0.36 mm. Cu, output 32.2 r/min, target to mid-mouse distance, 30 cm.

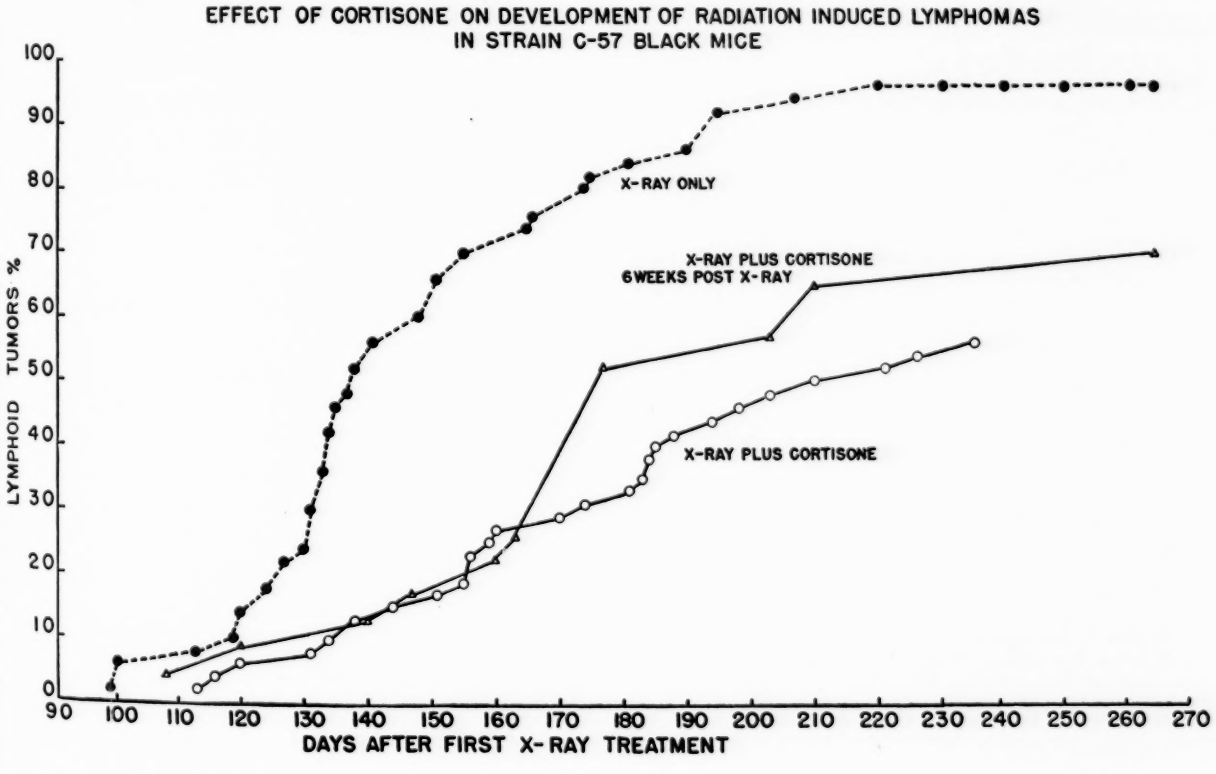


CHART 1

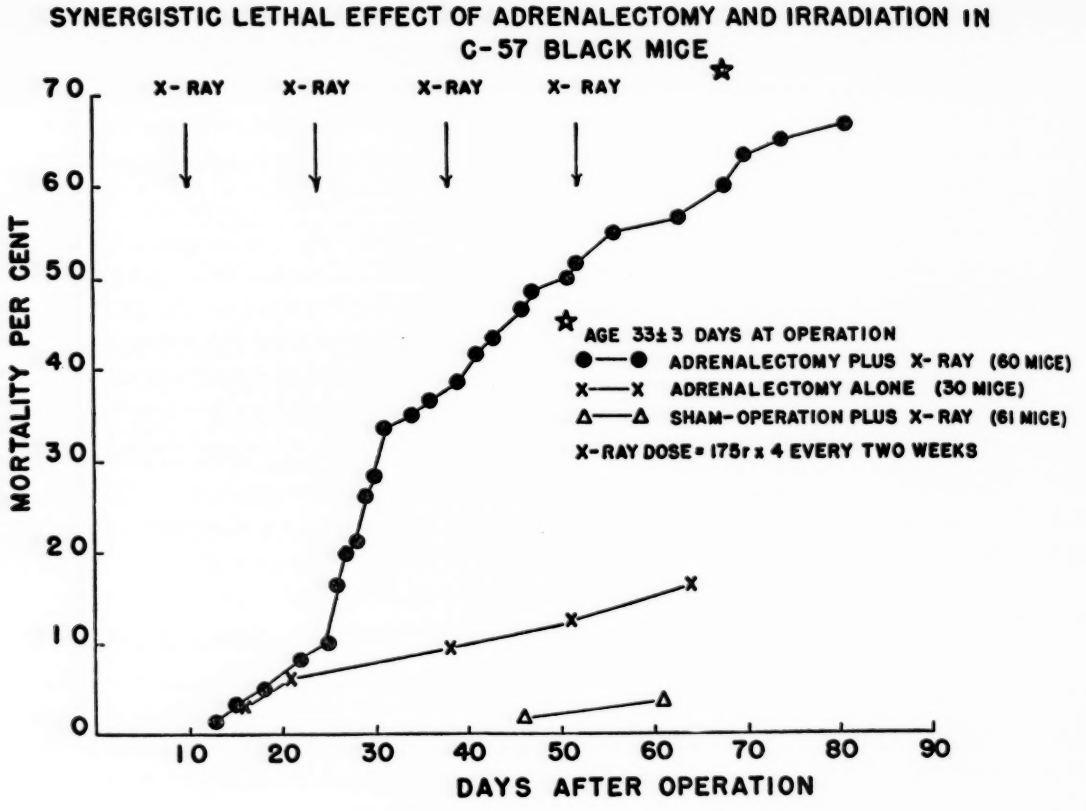


CHART 2

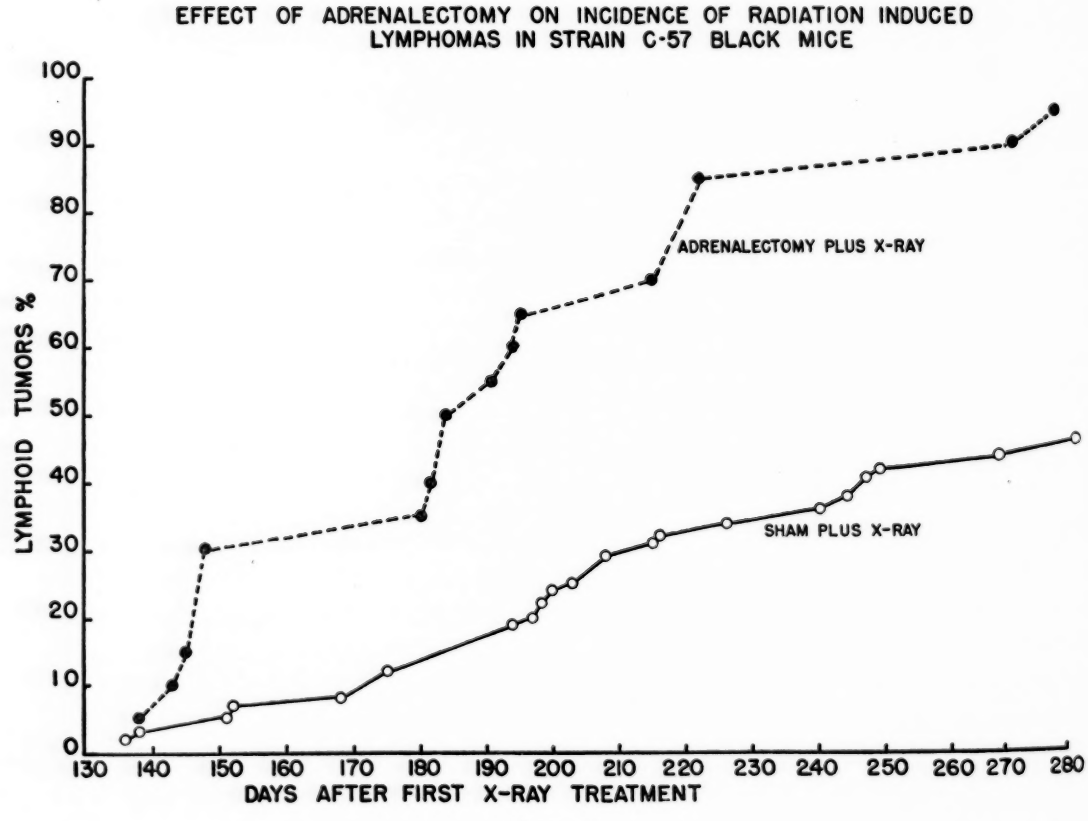


CHART 3

Of particular interest is the observation that similar inhibition occurs when cortisone injection is delayed until 6 weeks after the last x-ray treatment, indicating that the leukemogenic process may still be reversed or delayed at this time. This result is perhaps analogous to the effect of delayed administration of certain anticarcinogenic chemicals (2) or of delayed institution of caloric restriction (18) on chemically-induced skin tumor incidence. Berenblum (1) and Tannenbaum (19) have presented the thesis that tumor induction comprises a series of two or more stages which are to some degree mutually independent. Further study of this possibility in the case of radiation-induced lymphoid tumors seems desirable.

It has previously been reported that mortality among irradiated animals increases after adrenalectomy (3, 5) or hypophysectomy (15). In the present experiments, mice subjected to careful adrenal extirpation and maintained on an adequate dietary program suffered a profound increase in post-irradiation mortality. Since, under the treatment schedule employed, very few intact animals died, it would seem that irradiation acted as a stress test. It has been reported (17) that the lethal effect of massive x-ray doses in the LD_{50} range was no greater in adrenalectomized than in intact mice.

A statistical problem which has not been satisfactorily solved is the analysis of comparative lymphoid tumor incidence in the adrenalectomized-irradiated group, in which a high mortality occurred prior to the time at which tumors could be anticipated, and the sham operated-irradiated group in which deaths were few. It is difficult to ascertain that the surviving animals of the adrenalectomized-irradiated group reflect the degree of susceptibility to lymphoma induction of the initially established group as a whole. It would therefore seem desirable to repeat this experiment, utilizing maintenance doses of desoxycorticosterone acetate.

SUMMARY

1. The injection of cortisone either concurrently with or 6 weeks after total body irradiation significantly inhibited the development of lymphoid tumors in strain C57 black mice. No tumors occurred in mice treated with cortisone alone. Cortisone injection failed to modify the low lymphoma incidence observed after irradiation localized either over the mediastinum alone or over the whole body with the mediastinum shielded.

2. Bilaterally adrenalectomized strain C57 black mice exhibited a striking increase in mortality and a significant augmentation of lymphoid tumor incidence after total body irradiation.

3. Adrenal cortical extract and desoxycorticosterone acetate did not influence radiation-induced lymphoid tumor incidence under the experimental conditions employed.

REFERENCES

1. BERENBLUM, I. The Mechanism of Carcinogenesis: A Study of the Significance of Cocarcinogenic Action and Related Phenomena. Cancer Research, **1**:807–14, 1941.
2. CRABTREE, H. G. Influence of Bromobenzene on the Induction of SkinTumors by 3,4-Benzpyrene. Cancer Research, **4**:688–93, 1944.
3. CRONKITE, E. P., and CHAPMAN, W. H. Effect of Adrenalectomy on Radiation-induced Mortality of the Mouse. Proc. Soc. Exper. Biol. & Med., **74**:337–40, 1950.
4. DOUGHERTY, T. F., and WHITE, A. Influence of Adrenal Cortical Secretion on Blood Elements. Science, **98**:367–69, 1943.
5. EDELMANN, A. Adrenal Cortex and Survival of Rats after X-Irradiation. Fed. Proc., **9**:36, 1950.
6. GARDNER, W. U.; DOUGHERTY, T. F.; and WILLIAMS, W. L. Lymphoid Tumors in Mice Receiving Steroid Hormones. Cancer Research, **4**:73–87, 1944.
7. KAPLAN, H. S. Radiation-induced Lymphoid Tumors of Mice. Acta, de l'Union international contre le cancer (in press).
8. ———. Comparative Susceptibility of the Lymphoid Tissues of Strain C57 Black Mice to the Induction of Lymphoid Tumors by Irradiation. J. Nat. Cancer Inst., **8**:191–97, 1948.
9. ———. Preliminary Studies of the Effectiveness of Local Irradiation in the Induction of Lymphoid Tumors in Mice. *Ibid.*, **10**:267–70, 1949.
10. ———. Influence of Thymectomy, Splenectomy, and Gonadectomy on Incidence of Radiation-induced Lymphoid Tumors in Strain C57 Black Mice. *Ibid.*, **11**:83–90, 1950.
11. ———. Further Observations on Inhibition of Lymphoid Tumor Development by Shielding and Partial-Body Irradiation of Mice. Cancer Research, **11**:261, 1950.
12. LAW, L. W. Effect of Gonadectomy and Adrenalectomy on the Appearance and Incidence of Spontaneous Lymphoid Leukemia in C58 Mice. J. Nat. Cancer Inst., **8**:157–59, 1947.
13. LITTLE, C. C.; MURRAY, W. S.; and CLOUDMAN, A. M. The Genetics of Non-Epithelial Tumor Formation in Mice. Am. J. Cancer, **36**:431–50, 1939.
14. MARDER, S. N. Survival, Body Weights, and Lymphoid Tissue Weights Following Adrenalectomy in CBA Mice. J. Nat. Cancer Inst., **11**:133–39, 1950.
15. PATT, H. M.; SWIFT, M. N.; TYREE, E. B.; and STRAUBE, R. L. X-Irradiation of the Hypophysectomized Rat. Science, **108**:475–76, 1948.
16. REINHARDT, W. O., and HOLMES, R. O. Thymus and Lymph Nodes Following Adrenalectomy and Maintenance with NaCl in the Rat. Proc. Soc. Exper. Biol. & Med., **45**:267–70, 1940.
17. STRAUBE, R. L.; PATT, H. M.; TYREE, E. B.; and SMITH, D. E. Influence of Level of Adrenal Cortical Steroids on Sensitivity of Mice to X-Irradiation. Proc. Soc. Exper. Biol. & Med., **71**:539–41, 1949.
18. TANNENBAUM, A. The Dependence of the Genesis of Induced Skin Tumors on the Caloric Intake during Different Stages of Carcinogenesis. Cancer Research, **6**:673–77, 1944.
19. ———. The Importance of Differential Consideration of the Stages of Carcinogenesis in the Evaluation of Cocarcinogenic and Anticarcinogenic Effects. *Ibid.*, **4**:678–82, 1944.

Basal Metabolic Rate and Liver Tumors Due to Azo Dyes*

WILLIAM L. MILLER, JR., AND C. A. BAUMANN

(Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison, Wis.)

The question is still open whether hyperthyroidism alters the effectiveness of carcinogenic agents. Hyperthyroid agents have been given to mice or rats exposed to tar (10), 3,4-benzpyrene (1, 18), or 2-acetylaminofluorene (15), with conflicting effects on the rate or extent of tumor development reported. *A priori*, the hyperthyroid state might be expected to alter either the metabolism of the carcinogen, or the reactions leading toward the development of tumors, or still other reactions that tend to suppress them. The net effect would be a resultant of these factors and would vary with the degree of hyperthyroidism and with certain induced secondary effects, such as an altered net caloric intake. Similar considerations apply to the effect of the hypothyroid state on tumor development.

In the present study the basal metabolic rate (BMR) was altered in rats developing liver tumors due to *p*-dimethylaminoazobenzene (DAB) or m′-methyl-*p*-dimethylaminoazobenzene (m′ DAB), and this alteration was introduced either simultaneously with the carcinogen or alternately with it. The basal metabolic rates of the animals were measured at intervals, and parallel studies were made of the ability of various livers to destroy the carcinogen *in vitro*.

EXPERIMENTAL

Tumor Production in Rats with Abnormal Basal Metabolic Rates

Methods.—Adult, male albino rats were housed in screen-bottomed cages and were given food and water ad libitum. DAB was fed at the level of 0.06 per cent in Diet I, which contained: extracted casein, 12 per cent; salts, 4; corn oil, 5; and glucose monohydrate (Cerelose), to 100; and the following vitamins in mg/kg of diet: thiamine, 3.0; pyridoxine, 2.5; calcium pantothenate, 7.5; riboflavin, 1.0; and choline, 500. Diet II contained 18 per cent of casein and 0.1 per cent of cystine plus the following vitamins in mg/kg of diet: thiamine, 6.0; pyridoxine, 6.0; calcium pantothenate, 20; riboflavin, 1.0; nicotinic acid, 10; *p*-aminobenzoic acid, 300; inositol, 500; biotin, 0.5; folic acid, 0.5; and choline, 1,000. Each rat also received 2 drops of halibut liver oil every 4 weeks. Hyperthyroidism in the presence of DAB was usually induced by feeding 0.24 per cent of iodinated casein[1] in Diets I or II for 20 weeks; hypothyroidism was induced by 0.1 per cent of 2-thiouracil in the drinking water at pH 8.

For experiments with m′DAB, Diet III was used, which was like Diet I except that it contained 2 mg. of riboflavin and 1 gm. of choline per kilogram. The carcinogen and the metabolic drugs were fed alternately; 0.064 per cent of m′DAB was given for a total of 8 weeks, the feeding of the dye being interrupted after 4 weeks by a 4-week period during which dye-free diets were fed. Iodinated casein or 6-*n*-propyl-2-thiouracil[2] (PTU) were fed only during the "period of interruption" (5), which was followed by 4 weeks during which the dye was fed and a final 8-week period during which the diet was free from either metabolic drugs or dye.

Hyperthyroidism was produced by 0.25 per cent of iodinated casein in basal Diet III for 21 days, followed by a recovery period of 7 days. Hypothyroidism was produced by feeding 0.1 per cent of PTU for 8 days and 0.02 per cent for 18 days more. A near-normal BMR in the presence of the drugs was produced by the simultaneous feeding of 0.1 per cent of PTU and 0.1 per cent of iodinated casein in the basal diet for 8 days, followed by 0.02 per cent of PTU and 0.1 per cent of iodinated casein. These drugs were taken from the ration on the same day that the hypothyroid group started the recovery period. The consumption of oxygen by rats from the various groups was measured in an apparatus modified from that described by Schwabe and Griffith (17) and the results expressed as liters of oxygen consumed per 24 hours per square decimeter of body surface. The surface

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by the Jonathan Bowman Cancer Fund and by a grant from the Committee on Growth, American Cancer Society.

Received for publication April 26, 1951.

[1] Iodinated casein (Protamone), Cerophyl Laboratories, Inc., Kansas City, Mo.

[2] Generously supplied by Calco Chemical Division, American Cyanamid Co., Bound Brook, N.J.

area equals 11.36 $W^{2/3}$ (4), where W is the weight of the animal in kilograms.

Tumors in rats fed DAB.—The most obvious result of feeding DAB with iodinated casein was the poor condition of the animals; most of the hyperthyroid rats died before tumors developed, survival being only eight out of nineteen (Table 1, groups 2 and 5). Those that survived showed an over-all tumor incidence of 88 per cent, compared to 33 per cent in the controls. The use of the more complete Diet II did not effectively increase survival among the hyperthyroid animals (Table 1, groups 8 and 10), even though they were allowed to "recover" for a period of 2 weeks on a diet containing dye but no iodinated casein. In the hyperthyroid rats that survived, the incidence of tumors was 63 and 56 per cent, as compared to 53 and 36 per cent, respectively, in the control groups (Table 1, groups 7–10). 2-Thiouracil also appeared to increase tumor formation somewhat (Table 1, groups 3 and 6). Others have reported a decrease of liver tumors in rats fed both 2-thiouracil and DAB (7, 15).

TABLE 1

EFFECT OF ALTERED BASAL METABOLIC RATE ON CARCINOGENESIS IN RATS FED 0.06 PER CENT *p*-DIMETHYLAMINOAZOBENZENE*

Diet	Av. food intake gm/rat/day	BMR at wks. on dye l O_2/24 hr/dm² 4	11	13	15–20†	Survival at 20 wks.	Tumors at term	Neg. survivors at term	Per cent tumors
1.	14.3	1.59	1.62		1.66	8/8	3	5	38
2. Iodinated casein‡	17.6	3.90	4.78			4/9	3	1	75
3. 2-Thiouracil	11.2	1.53	1.30		1.14	8/8	6	2	75
4.	15.2	1.57	1.63		1.63	7/7	2	5	29
5. Iodinated casein	19.7	3.71	4.45			4/10	4	0	100
6. 2-Thiouracil	14.7	1.63	1.39		1.17	7/7	4	3	57
7.	16.1					15/15	8	7	53
8. Iodinated casein	19.7		4.20	1.67§	4.02	8/20	5	3	63
9. High vitamins 0.10 per cent cystine	15.9					14/15	5	9	36
10. High vitamins 0.10 per cent cystine Iodinated casein	20.5		4.40	1.67§	3.80	9/20	5	4	56

* *p*-Dimethylaminoazobenzene (DAB) fed all groups for 20 weeks followed by 8 weeks of dye-free basal diet.

† Groups 1–6 read at 20 weeks; groups 7–10 at 15 weeks.

‡ 0.480 per cent Iodinated casein for 5 weeks followed by 15 weeks at 0.24 per cent. All other hyperthyroid rats were fed 0.24 per cent of iodinated casein.

§ BMR recovery from 11th to 13th week.

The effect of an altered BMR in the absence of carcinogen.—During the period of interruption, the BMR of rats fed iodinated casein increased steadily over a 21-day period (Table 2), advancing from 1.55 to 3.83 l O_2/24 hrs/dm², an increased oxygen consumption of 146 per cent. Eight days of recovery decreased the BMR to the normal level, and the average increase in BMR during the dye-free period was approximately 75 per cent (Table 2). Food consumption increased during hyperthyroidism, and a small increase in weight was also observed (Table 3, groups 2 and 6). In two separate series hyperthyroidism increased the number of tumors under these conditions: 50 per cent of the hyperthyroid rats developed liver tumors, as compared to 23 or 27 per cent for the controls (Table 3, groups 1 and 2, 5 and 6).

The BMR of rats fed 0.1 per cent of PTU for 8 days decreased from 1.55 to 1.12 l O_2/24 hrs/dm², a 28 per cent depression from the normal (Table 2); 0.02 per cent of PTU then maintained the hypothyroid state for 18 days. Four days of recovery increased the BMR to a normal level of 1.50 l O_2/24 hrs/dm². Hypothyroid rats consumed about the same amount of food as the controls, and the final incidence of tumors was also essentially the same as in the control groups: 20–27 per cent (Table 3, groups 1 and 3, 5 and 7).

The BMR of rats fed both 0.1 per cent of iodinated casein and 0.1 per cent of PTU for 8 days was 1.73 l O_2/24 hrs/dm², and the subsequent feeding of 0.1 per cent of iodinated casein with 0.02 per cent of PTU resulted in a BMR only slightly above normal (Table 2). Under these conditions the final incidence of liver tumors was also essentially normal (Table 3, groups 1, 4, 5, and 8).

DESTRUCTION OF DAB BY LIVER FROM RATS WITH ABNORMAL BMR'S

Methods.—Adult male rats were fed Diet III plus either iodinated casein or PTU, as in the tumor experiments. In addition, one of the hyperthyroid groups received a so-called "high vitamin diet" which was like Diet III except that it contained 24 per cent of casein and 20 μg/gm each of riboflavin and nicotinic acid. Animals were decapitated at intervals, and measurements were

made of the concentration of riboflavin (2, 6) in the liver and of the ability of liver slices to destroy DAB (8, 11). For certain slices the consumption of oxygen was measured in a conventional Warburg apparatus. Corresponding basal metabolic rates were measured on other animals from the same groups.

The livers were placed on ice immediately after removal from the animal, and slices were prepared with a razor blade and 2 pieces of frosted glass (19); 200 mg. (wet) of the slices was added to 1.9 ml. of Krebs-Ringer-phosphate solution containing 100 μg. of DAB in 0.1 ml. of ethanol (8). The flasks were incubated by being shaken in a water bath at 37° C. and the rate of dye destruction measured after 5 minutes of equilibration.

TABLE 2

CHANGES IN BASAL METABOLIC RATE DURING THE PRECANCEROUS PERIOD*

Feeding sequence	Days on diet	1 Control BMR† 1.6±.05	2 Hyper BMR† 1.6±.05	3 Hypo BMR† 1.6±.05	4 "Hyper-Hypo" BMR† 1.6±.05
0.064 per cent *m*'DAB	12	1.55	(1.55)	(1.55)	(1.55)
	28				
Interrupted basals (no dye)‡	4		2.19	1.21	1.53
	5		2.57	1.25	1.56
	7		2.63	1.23	
	8			1.12	1.73
	10		3.24		1.87
	14		3.40	1.15	1.77
	17			1.09	1.86
	18	1.60	3.68		
Hyper recovery	21		3.83		
Hypo and "Hyper-Hypo"	26		2.55	1.03	
	27				1.96
recovery	28		2.12	1.08	1.74
0.064 per cent *m*'DAB	2	1.60	1.54	1.50	
	7		1.66		1.66
	28		1.53	1.50	1.50

* Weights, food intake, and tumor data in Table 3.

† Basal metabolic rate, liters O_2/24 hrs/dm²; each figure is an average for two or more animals. The reading for each animal was an average of two or more readings. Recovery data were obtained on the same animals which had shown maximum changes in BMR.

‡ Drug levels fed in the period of interruption were:
Hyper, 0.25 per cent iodinated casein (Protamone) 21 days, basal alone 7 days.
Hypo, 0.1 per cent 6-*n*-propyl-2-thiouracil (PTU) 8 days, 0.02 per cent PTU 18 days, basal alone 2 days.
Hyper+Hypo, 0.1 per cent iodinated casein and 0.1 per cent PTU for 8 days, 0.1 per cent iodinated casein and 0.02 per cent PTU for 18 days, basal alone 2 days.

The destruction of DAB by liver homogenates was measured by a slight modification of a previous method (13). Homogenates were prepared by grinding 1 gm. of fresh liver with a Potter-Elvehjem pestle in ice-cold water to give a 10 per cent mixture, and aliquots were transferred to cold 25-ml. Erlenmeyer flasks containing the other required ingredients. A typical mixture contained the following, adjusted to pH 7.4: 0.4 ml. of homogenate, 0.4 ml. of 0.03 M hexose diphosphate,[3] 0.2 ml. of 0.6 M nicotinamide, 0.1 ml. of 0.1 M $MgCl_2$, 0.4 ml. of 0.5 M KCl, 0.5 ml. of 0.1 M K_2HPO_4-KH_2PO_4 buffer, and 0.1 ml. of an ethanol solution of DAB (60 μg.), and water to a final volume of 3.0 ml. Homogenate flasks were incubated for 30 minutes.

The enzymatic activity in all tissue systems was stopped by the addition of 0.2 ml. of 11 N KOH, and the dye was recovered with 5 ml. of methanol and then extracted into 10 ml. of Skelly Solve B, which formed a clear solution after a few minutes of standing. A 1-ml. aliquot of this upper layer was transferred to a standardized Evelyn tube, 10 ml. of 7 N HCl was added, and, after gentle rotation, the dye was transferred into the lower acid phase where it could be read in the Evelyn colorimeter. The colorimeter constant for DAB in 7 N HCl at 515 mμ was found to be 29.2. "Zero time" incubation flasks gave recoveries of 98 to 101 per cent.

Destruction of DAB by liver slices.—Others have shown that the ability of rat liver to destroy DAB depends on the level of riboflavin fed to the animal (9, 14). In the present studies, 100 mg. of liver slices from normal rats fed 2 μg. of riboflavin/gm of diet (as in the tumor studies) destroyed 13–14 μg. of dye per hour (Tables 4 and 5); but when the basal diet contained 12 μg. of riboflavin/gm or or more, dye destruction was 20–26 μg/hour. This latter rate was not altered by feeding additional protein or other vitamins (Table 5).

Hyperthyroidism markedly lowered the ability of the liver slices to destroy DAB. A decrease was evident after only 1 week on the hyperthyroid diet, while after 3 weeks slices from hyperthyroid rats fed Diet III destroyed only 2–3 μg. of dye/100 mg/hour, as compared to 13–14 μg. for the control slices (Table 4). Hyperthyroidism likewise impaired dye destruction by livers of rats fed diets high in riboflavin; the effect was delayed slightly but was equal in magnitude to that observed on less complete diets (Tables 4 and 5). The further addition to the diet of factors such as 5 per cent of whole liver[4] or 30 μg. of vitamin B_{12}/kg, which prolong the lives of young hyperthyroid rats (3), likewise delayed but did not prevent the loss in ability of the hyperthyroid liver slice to destroy DAB (Table 5, groups 6 and 7).

The hyperthyroid livers contained at least as much riboflavin as livers from normal or hypothyroid animals (Table 4), and they also absorbed approximately normal amounts of O_2 during the first 45 minutes of incubation in Krebs-Ringer-

[3] Hexose-1,6-diphosphate regenerated from the Ba salt, Schwarz Laboratories, Inc., New York.

[4] Wilson Laboratories, Chicago, Ill.

TABLE 3

LIVER TUMORS DUE TO *m'*DAB: EFFECT OF IODINATED CASEIN AND 6-*n*-PROPYL-2-THIOURACIL (PTU) DURING THE PERIOD OF INTERRUPTION

	Diet* during period of interruption	Average wt. 0 wks. (gm.)	Average wt. 4 wks. (gm.)	Food intake on dye-free diet gm/rat/day	Average wt. 8 wks. (gm.)	Average wt. 12 wks. (gm.)	Food intake on dye gm/rat/day	Survival at 12 wks.	Tumors at 20 wks.	Neg. survivors at 20 wks.	Per cent tumors
1. Control	Basal	246	201	14.1	274	239	9.5	13/15	3	10	23
2. Hyper	I. Casein	241	186	17.2	220	228	10.7	12/15	6	6	50
3. Hypo	PTU	242	190	14.0	270	253	10.7	14/15	3	11	21
4. "Hyper-Hypo"	I. Casein + PTU	244	190	15.4	234	236	9.7	11/15	3	8	27
5. Control	Basal	277	235	18.4	291	269	14.1	15/15	4	11	27
6. Hyper	I. Casein	278	223	21.5	249	267	12.8	12/15	6	6	50
7. Hypo	PTU	274	224	17.5	272	263	12.9	15/15	3	12	20
8. "Hyper-Hypo"	I. Casein + PTU	280	220	19.6	250	255	13.7	14/15	4	10	29

* Hyperthyroid rats fed 0.25 per cent iodinated casein for 3 wks. Recovery 1 wk. Hypothyroid rats fed 0.10 per cent 6-*n*-propyl-2-thiouracil for 1 wk., 0.02 per cent PTU for 2½ wks.; recovery ½ wk. Hyperthyroid+hypothyroid rats fed 0.1 per cent I. casein+0.1 per cent PTU for 1 wk., 0.1 per cent I. casein+0.02 per cent PTU 2½ wks. Rate of change of basal metabolic rate during "period of interruption" in Table 2. All groups fed 0.064 per cent *m'*-methyl-*p*-dimethylaminoazobenzene (*m'*DAB) for 4 wks.; various basal diets for 4 wks.; 0.064 per cent *m'*DAB for 4 wks.; basal diet III for 8 wks.

TABLE 4

DAB DESTROYED BY LIVER SLICES FROM RATS WITH VARIOUS BASAL METABOLIC RATES*

Days on diet	0	7	14	21	28
	μg.	μg.	μg.	μg.	μg.
1. Control diet III	13.5	13–14	13.5	13–14	13–14
2. Hyper		5–11	3–5	2–3	3
3. Hyper†		10–14	6–7	5–6	
4. Hypo			7–12	9	11–12
5. "Hyper-Hypo"			14–15	13–15	15–16

CORRESPONDING LIVER RIBOFLAVIN VALUES, AND BMR'S‡ OF THE RATS

	B_2 μg/gm	BMR	B_2 μg/gm	BMR	B_2 μg/gm	BMR	B_2 μg/gm	BMR	B_2 μg/gm	BMR
1. Control diet III	27.0	1.61			21.2	1.61	23.7		21.7	(1.60)
2. Hyper			26.7	2.35	25.8	3.44	29.1	3.60	27.1	(3.75)
3. Hyper†			30.8	2.23	31.5	3.49	29.4	3.45		
4. Hypo					24.6	1.17			26.9	(1.13)
5. "Hyper-Hypo"					23.9	1.85	25.8	1.88	25.7	(1.84)

* Ranges given in μg. DAB destroyed/100 mg tissue (wet)/hr and including average values for 3 or more separate livers.

† High casein, riboflavin, and nicotinic acid.

‡ BMR values are in liters O_2/24 hrs/dm² body surface.

The numbers in parentheses are BMR values from previous experiments with these diets.

TABLE 5

DESTRUCTION OF DAB BY LIVER SLICES FROM RATS FED DIETS THAT FAVOR RIBOFLAVIN RETENTION OR THAT TEND TO COUNTERACT HYPERTHYROIDISM

	Normal				Hyperthyroid			
	Weights		DAB destr.*		Weights		DAB destr.*	
WEEKS ON DIET	0 (gm.)	3 (gm.)	2	4	0 (gm.)	3 (gm.)	2	4
1. 12 per cent Casein, 2 μg B_2/gm			13	13			6	4
2. 12 per cent Casein, 20 μg B_2/gm			20	26			12	5
3. 24 per cent Casein, 2 μg B_2/gm			14	16			7	6
4. 24 per cent Casein, 20 μg B_2/gm	167	219	24	23	163	163	10	9
5. 22 per cent Casein, 12 μg B_2/gm	166	215	23	24	163	171	12	10
6. 22 per cent Casein, 12 μg B_2/gm 5 per cent whole liver powder	165	230	26	26	164	186	14	11
7. 22 per cent Casein, 12 μg B_2/gm Vitamin B_{12}	165	240	26	25	167	198	16	10

* DAB destruction given as μg/100 mg wet weight/hr.

phosphate solution (12). At 60 minutes, however, the Q_{O_2} of the hyperthyroid slices was 4.1, as compared to 5.0 for slices from normal rats. Added glucose failed to increase either the Q_{O_2} or the ability of the slices to destroy dye.

Slices from hypothyroid rats showed a somewhat decreased rate of dye destruction (Table 4); those from rats receiving both iodinated casein and PTU destroyed the dye at a normal rate.

Destruction of DAB by homogenates.—A system containing only the liver homogenate and certain inorganic ions failed to destroy the dye (Table 6, group 1), and the addition of hexose diphosphate (HDP) was also ineffective (Table 6, group 2).

TABLE 6

DESTRUCTION OF DAB BY RAT LIVER HOMOGENATES
μG DAB DESTROYED PER FASK IN 30 MIN.
(40 mg. Tissue Wet Wt.)*

Supplements†	Control	Hyperthyroid
1. 0.003 M $MgCl_2$, 0.07 M KCl 0.02 M Phosphate buffer (ions)	4 (3–5)	3 (1–4)
2. 0.004 M Hexose diphosphate plus ions	4 (1–9)	4 (1–9)
3. 0.04 M Nicotinamide plus ions	27 (21–32)	8 (4–10)
4. 0.04 M Nicotinamide plus ions plus 0.0006 M ATP	20 (16–28)	9 (8–10)
5. 0.04 M Nicotinamide plus 0.004 M hexose diphosphate plus ions	38 (34–43)	37 (33–41)
6. 0.04 M Nicotinamide plus either 0.04 M citrate, succinate, fumarate, or malate plus ions	33 (25–40)	31 (25–35)
Liver slices (ug DAB destroyed/100 mg wet wt/hr)	26 (24–28)	8 (6–9)

* Range values include average dye destruction values for 3–5 separate livers.

† Molarity in final concentration. Volume of each flask maintained at 3 ml. with distilled water.

When nicotinamide and inorganic ions were added to a normal liver homogenate, moderate dye destruction occurred, varying from 21 to 32 μg. of DAB per flask, but hyperthyroid homogenates under the same conditions destroyed only 4–10 μg. of DAB (Table 6, group 3). The further addition of HDP produced moderate increases in the destruction of dye by homogenates from normal livers and very marked increases by the hyperthyroid homogenates (Table 5, group 5). In the presence of HDP and nicotinamide, the destruction of dye was essentially equal for the two types of homogenates, 37–38 μg. of DAB. The further addition of either diphosphopyridine nucleotide[5] or flavin-adenine dinucleotide[6] to the system used in group 5 did not produce significant changes in the rate of dye destruction.

Citrate, succinate, fumarate, and malate, which stimulate DAB destruction by normal liver homogenates (13), also increased dye destruction by hyperthyroid homogenates (Table 6, group 6). Single or successive additions of adenosine triphosphate[7] (ATP) in place of HDP failed to increase the destruction of dye by either control or hyperthyroid homogenates. The addition of HDP to hyperthyroid slices also increased the rate of dye destruction (12), but the "hyperthyroid effect" was not reversed as completely in the slices as in the homogenates. These experiments are consistent with the view that the defect in the hyperthyroid slice is in metabolizable carbohydrate rather than in the enzymes or coenzymes involved in the destruction of DAB.

DISCUSSION

While the present experiments show that the incidence of liver tumors may be increased by feeding iodinated casein to suitably precancerous rats, they do not indicate the mechanism by which this is accomplished. When the hyperthyroid state is induced in rats receiving DAB, there are at least two secondary factors that favor tumor development: the rats eat more food and therefore more carcinogen, and there is a decrease in the ability of the liver to destroy the dye. This latter factor cannot be stated in exact terms, for the rate of dye destruction by the surviving liver slice may not parallel that in the living animal. Obviously, however, any decrease in the ability of the animal to destroy the dye *in vivo* would be equivalent to exposure of the liver to more carcinogen.

When the hyperthyroid state is induced in the absence of carcinogen (during the middle period), these particular complications are avoided. During the second period of dye feeding, livers from rats that had been hyperthyroid destroyed DAB at rates similar to those for rats that had remained normal during the middle period. But there is another difficulty. Rats that had been hyperthyroid maintained their weights or increased them slightly when the carcinogen was fed for the second time (Table 3); "normal" rats, on the other hand, lost weight on the second application of the dye. Thus, livers from the hyperthyroid groups may have been increasing in size while the carcinogen was applied, similar to the increases observed in rats that had been restricted in calories during a middle period (5). These latter rats showed an increased incidence of liver tumors (5) presumably because a carcinogen is particularly effective when applied to proliferating tissue (16), and a similar mechanism might be responsible for part of the increase in tumors in the hyperthyroid groups 2 and 6

[5] Schwarz Laboratories, Inc., New York.

[6] Obtained from Philip Feigelson of this department.

[7] Sigma Chemical Co., St. Louis, Mo.

(Table 3) in the present study. However, rats that received amounts of propyl thiouracil and iodinated casein during the middle period to yield a near-normal BMR showed weight changes very similar to those in the hyperthyroid groups (Table 3, groups 2 versus 4, 6 versus 8), yet developed the same number of tumors as those in the control groups. The proliferative effect in the so-called "hyper-hypo" group must have been similar to that in the hyperthyroid animals which developed the greater number of tumors. Hence, the possibility remains that an increased BMR may itself augment the processes that lead to the formation of liver tumors. The effect, however, is not particularly large.

SUMMARY

1. Rats were fed 0.064 per cent of m′ methyl-*p*-dimethylaminoazobenzene for 4 weeks, followed by 4 weeks during which certain dye-free diets were fed which altered the basal metabolic rate; the dye was then fed for another 4 weeks. Other rats were fed 0.06 per cent of *p*-dimethylaminoazobenzene (DAB) for 20 weeks in diets which altered the BMR.

2. Hyperthyroid animals receiving DAB usually failed to survive, but the incidence of tumors in the survivors was high. Hyperthyroidism induced in precancerous rats in the absence of azo dye also increased tumor incidence. Thiouracil appeared to increase tumor incidence in rats fed DAB, but the more potent depressant, 6-*n*-propyl-2-thiouracil, failed to affect a development of tumors when given during the period of interruption.

3. Liver slices from hyperthyroid rats destroyed less than one-fourth as much DAB as slices from normal rats. Slices from rats fed high levels of riboflavin destroyed nearly twice as much dye as slices from control rats, but when the rats which received high riboflavin were made hyperthyroid, the ability to destroy the dye decreased, although liver riboflavin remained high.

4. In media containing inorganic salts and nicotinamide, homogenates from normal livers destroyed the dye at a high rate, while homogenates from hyperthyroid livers failed to destroy much dye. Homogenates from hyperthyroid livers destroyed approximately normal amounts of dye when hexose diphosphate was added to the medium.

REFERENCES

1. ANARDI, T. L'azione della follicolina, tiroxina ed endotimina sul sarcoma da benzopirene. Nota riassuntiva. Med. sper. arch. ital., **8**:181–82, 1941. "Abstr.," Ztschr. f. Krebsforsch., **52**:84, 1942.
2. ANDREWS, J. S. A Collaborative Study of Riboflavin Methods. Cereal Chem., **20**:3–23, 1943.
3. BETHEIL, J. J., and LARDY, H. A. Comparative Effectiveness of Vitamin B_{12}, Whole Liver Substance and Extracts High in APA Activity, as Growth Promoting Materials for Hyperthyroid Animals. J. Nutrition, **37**:495–509, 1949.
4. CARMAN, G. G., and MITCHELL, H. H. Estimation of the Surface Area of the White Rat. Am. J. Physiol., **76**:380–84, 1926.
5. CLAYTON C. C., and BAUMANN, C. A. Diet and Azo Dye Tumors: Effect of Diet during a Period When the Dye is Not Fed. Cancer Research, **9**:575–82, 1949.
6. CONNER, R. T., and STRAUB, G. J. Combined Determination of Riboflavin and Thiamine in Food Products. Indust. & Engin. Chem. (Anal. Ed.), **13**:385–88, 1941.
7. HARRIS, P. N., and CLOWES, G. H. A. The Effect of Thiosalicylic Acid, Thiouracil, and Folic Acid upon *p*-Dimethylaminoazobenzene Carcinogenesis. Cancer Research, **9**:565, 1949.
8. KENSLER, C. J. The Influence of Diet on the Ability of Rat-Liver Slices to Destroy the Carcinogen N, N-Dimethyl-*p*-Aminoazobenzene. Cancer, **1**:483–88, 1948.
9. ———. The Influence of Diet on the Riboflavin Content and the Ability of Rat Liver Slices to Destroy the Carcinogen N,N-Dimethyl-*p*-Aminoazobenzene. J. Biol. Chem. **179**:1079–84, 1949.
10. KREYBERG, L. Influence of Dinitrokresol on the Development of Tar Tumors in Mice. Am. J. Cancer, **36**:51–55, 1939.
11. MILLER, J. A., and BAUMANN, C. A. The Determination of *p*-Dimethylaminoazobenzene, *p*-Monomethylaminoazobenzene, and *p*-Aminoazobenzene in Tissue. Cancer Research, **5**:157–61, 1945.
12. MILLER, W. L., JR. Effect of Basal Metabolic Rate on Tumor Formation with Azo Dyes. M.S. Thesis, University of Wisconsin, 1950.
13. MUELLER, G. C., and MILLER, J. A. The Metabolism of 4-Dimethylaminoazobenzene by Rat Liver Homogenates. J. Biol. Chem., **176**:535–44, 1948.
14. ———. The Reductive Cleavage of 4-Dimethylaminoazobenzene by Rat Liver: Reactivation of Carbon Dioxide-Treated Homogenates by Riboflavin-Adenine Dinucleotide. *Ibid.*, **185**:145–54, 1950.
15. PASCHKIS, K. E.; CANTAROW, A.; and STASNEY, J. The Influence of Thiouracil on Carcinoma Induced by 2-Acetaminofluorene. Cancer Research, **8**:257–63, 1948.
16. ROUS, P., and KIDD, J. G. Conditional Neoplasms and Subthreshold Neoplastic States: A Study of Tar Tumors of Rabbits. J. Exper. Med., **73**:365–90, 1941.
17. SCHWABE, E. L., and GRIFFITH, F. R., JR. An Easily Constructed Rat Metabolism Apparatus Which Automatically Records Oxygen Consumption and Animal Activity. J. Nutrition, **15**:187–98, 1938.
18. TANNENBAUM, A., and SILVERSTONE, H. Effect of Low Environmental Temperature, Dinitrophenol, or Sodium Fluoride on the Formation of Tumors in Mice. Cancer Research, **9**:403–10, 1949.
19. UMBREIT, W. W.; BURRIS, R. H.; and STAUFFER, J. F. Manometric Techniques and Tissue Metabolism, pp. 110–19. Minneapolis: Burgess Publishing Co., 1949.

The Synthesis of 3,4-Benzpyrene-5-C^{14} and of 2-Acetylaminofluorene-9-C^{14}*†

CHARLES HEIDELBERGER AND HELGA STRAUBE RIEKE

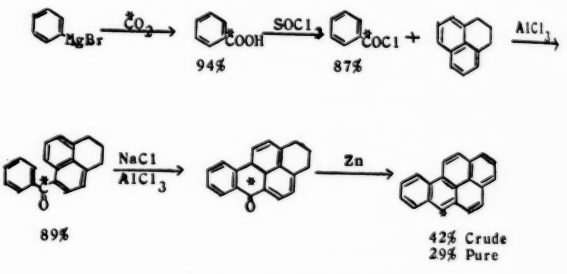
(McArdle Memorial Laboratory, Medical School, University of Wisconsin, Madison 6, Wis.)

The introduction of the tracer technic as a new tool for the elucidation of the metabolism of carcinogens has provided an impetus for reinvestigations and new researches in this field. The first compound to be so used was 1,2,5,6-dibenzanthracene-9,10-C^{14}(6), and considerable work has been devoted to a study of its metabolism (7–10, 17). Shortly thereafter, 20-methylcholanthrene-11-C^{14} was synthesized (2), and its distribution in the mouse was investigated (3). 3,4-Benzpyrene is an extremely powerful and rapidly acting carcinogenic hydrocarbon, and its chemical and biological properties, especially its metabolic behavior, have been investigated very thoroughly. These studies depended on changes observed in the fluorescence and ultraviolet spectra during the course of metabolism. We decided to label this compound with radioactive carbon in order to provide a more critical test of the advantages of the tracer technic over optical methods than has been obtained with the other compounds thus far studied, because of the considerable body of information already available about the metabolism of benzpyrene.

Another interesting carcinogen, 2-acetylaminofluorene, has been labeled by Ray and Geiser (16), and its metabolism is being investigated by Morris and the Weisburgers (15). Because of the relatively low specific activity of the 2-acetylaminofluorene-9-C^{14} prepared by Ray and Geiser, it seemed important to us to undertake the preparation of the compound with considerably higher specific activity, so that certain projected metabolic experiments might be undertaken. The synthetic scheme of Ray and Geiser (16) was followed, but the improvement in yield in several of the steps was so significant that we are prompted to report the results of our synthesis at the present time.

The synthesis of 3,4-benzpyrene-5-C^{14} was carried out by the method of Fieser and Hershberg (5), and with the yields indicated:

Synthesis of 3,4-Benzpyrene-5-C^{14}



Overall yield = 22%

Considerable experimentation was required to perfect conditions for the synthesis on a small scale before the requirements of high yields, conditional for high specific activity, could be met. It was shown that the conditions for each reaction must be controlled very carefully, as specified in the experimental descriptions. Furthermore, the starting material, perinaphthane, had to be carefully purified before it was suitable for use in the subsequent reactions. This compound was prepared by catalytic hydrogenation of perinaphthanone-7, which was synthesized by the method of Fieser and Gates (4). This reduction has not been previously carried out, since Fieser and Hershberg (5) prepared the perinaphthane by catalytic hydrogenation of perinaphthenone. This represents a significant improvement, since higher yields are obtained by this procedure, both in the synthesis of the perinaphthanone and in its hydrogenation, than by the earlier method (5).

Because small amounts of highly radioactive impurities cannot be tolerated in tracer experiments, it was necessary to purify the benzpyrene with great care. It was found that distillation, chromatography twice on activated alumina, and crystallization were required before the carcinogen was obtained sufficiently pure for the metabolic experiments.

* An abstract of part of this report appeared in Cancer Research, 11:255, 1951.

† This work was supported in part by a grant from the Wisconsin Section of the American Cancer Society.

Received for publication April 26, 1951.

EXPERIMENTAL

3,4-BENZPYRENE-5-C^{14}

Benzoic acid-carboxyl-C^{14}.—Radioactive benzoic acid was prepared by carbonation of phenyl magnesium bromide, as described in "Isotopic Carbon," p. 180 (1). The Grignard reagent, prepared from 0.6 gm. of magnesium and 3.9 gm. of bromobenzene in anhydrous ether, was shown by titration to contain 0.63 millimoles per milliliter. The carbonation reaction was carried out with 20 ml. (12.8 mM) of Grignard reagent and 1.953 gm. (10.0 mM) of barium carbonate containing 7 mc. of radioactivity. After hydrolysis, the ethereal solution was exhaustively extracted with dilute NaOH, the aqueous layer washed with ether, acidified, extracted with ether and re-extracted with a small volume of NaOH. On acidification of the hot solution, followed by cooling and filtration, 1.036 gm. of benzoic acid was obtained. The mother liquor was extracted with ether, and the ether layer extracted with alkali. On acidification, an additional 103 mg. was obtained. Total yield: 1.139 gm. (94 per cent), m.p. 119°–121°.

Benzoyl chloride-carbonyl-C^{14}.—Purified thionyl chloride, 1.8 ml. (25 mM) was added to a solution of the benzoic acid in 10 ml. of anhydrous benzene, and the mixture was refluxed on the steam bath for 3 hours, by which time the evolution of hydrogen chloride had ceased. After removal of the benzene *in vacuo*, the thionyl chloride was completely eliminated by two successive additions and vacuum distillations of benzene. Finally, the benzoyl chloride was distilled *in vacuo*, and a yield of 1.126 gm. (87 per cent) of colorless benzoyl chloride was obtained.

Perinaphthane.—Perinaphthanone-7 was prepared by the method of Fieser and Gates (4) and was hydrogenated in the Parr apparatus at 2 atmospheres over a 30 per cent palladium-on-charcoal catalyst (14). Attempted reductions by the Wolff-Kischner (11) and Clemmensen procedures led either to intractible tars or to perinaphthanol-7. The solvent used was acetic acid containing a small amount of perchloric acid as an activator (12). In a typical experiment, to a solution of 2 gm. of recrystallized perinaphthanone-7 in 25 ml. of glacial acetic acid were added 1 gm. of catalyst and 2 ml. of 70 per cent perchloric acid. The hydrogenation was complete in 2 hours. The catalyst was filtered, the acetic acid distilled *in vacuo*, and the product was distilled. A yellowish after-run (perinaphthanol) was obtained, and the main product distilled at 80°–85° at 0.1 mm.; a considerable tarry residue remained. The perinaphthane fraction was crystallized 3 times from dilute methanol, and a yield of 0.851 gm. (46 per cent) of large colorless leaflets was obtained. The melting point of the pure compound is 68.5°–69.0°. (Fieser and Hershberg [5] report 65°–65.5°.) The picrate (orange-red needles from methanol) melts at 150°–151°, the trinitrobenzoate (yellow needles from methanol) melts at 158°–159°, in agreement with the literature (5).

3-Benzoylperinaphthane-carbonyl-C^{14}.—The Perrier complex was prepared by the slow addition of 1.078 gm. of fresh aluminum chloride to 1.126 gm. of the labeled benzoyl chloride. During the addition the mixture was warmed and finally heated gently over a free flame until all solid particles had disappeared. A solution of purple melt in 15 ml. of carbon disulfide was added slowly from a warmed separatory funnel to a warm solution of 1.295 gm. of perinaphthane in 5 ml. of carbon disulfide. The mixture was refluxed gently for ½ hour, and the cooled solution was decomposed with ice and dilute hydrochloric acid. Following evaporation of the carbon disulfide, the oily ketone was extracted thoroughly into ether and was then washed several times with water and dried. The ether was evaporated on the steam bath, and the residue was subjected to short-path distillation ("Kugelrohr") *in vacuo*. A small fore-run was collected, the main product distilled at 160°–180° at 0.01 mm., and was immediately redistilled. A yield of 1.92 gm. (89 per cent) of light-yellow, viscous, oily ketone was obtained. This material was sufficiently pure to be used directly in the succeeding steps of the synthesis. The oil could be crystallized from petroleum ether and melted at 62°–63°.

3,4-Benzpyrene-5-C^{14}.—A melt was made from a mixture of 10.15 gm. of fresh aluminum chloride and 2.92 gm. of anhydrous sodium chloride, and poured onto 1.92 gm. of the radioactive benzoylperinaphthane in a 25-cc. Erlenmeyer flask. The flask was stoppered loosely and heated in an oil bath for 3 hours at 130°–140° and for 2 more hours at 145°–150°. The reaction mixture was decomposed with ice and dilute hydrochloric acid, and the brown product was filtered, washed with water, and collected. Considerable effort and care were required in order to effect complete decomposition. It was necessary to scrape the flask contents with a spatula to insure contact of the aqueous phase with the gummy solid. Rubber gloves were worn during these manipulations. During the filtration a small amount of dark brown oil passed through the paper and was discarded, since it had been shown in practice experiments that it was not converted into benzpyrene.

The solid product and the filter paper were thoroughly macerated and transferred to a small

distillation flask with a wide path to a sealed-on receiver. Once in the flask, it was mixed with 31.8 gm. of zinc dust. The flask was placed in a salt bath with the receiver insulated from the electrical heat source by a Masonite screen. The reaction mixture was heated to a very high temperature, and the benzpyrene was distilled rapidly into the receiver, which was cooled with wet cotton. The temperature necessary was greater than 600°, and the distillation was usually complete in 20 minutes. Further heating or attempted vacuum distillation failed to increase the yield. If the distillation was carried at too low a temperature or too slowly, a decreased yield was obtained.

The orange-brown product was dissolved in a small amount of benzene and chromatographed immediately on a 2.5×40 cm. column of activated alumina. The solvent mixture was 1:1 purified benzene and ligroin (Skellysolve B). Because of the light-sensitivity of benzpyrene, the chromatography was carried out in darkness and the progress determined by occasional short examinations with an ultraviolet lamp. A band of yellow-fluorescent impurities was eluted first, followed by the benzpyrene (blue-fluorescent). A large band of yellow-fluorescent impurities remaining on the column was eluted with alcohol. When the two yellow impurities were recovered from the solvents and subjected to zinc dust distillation in the same way as before, a small amount of additional benzpyrene could be obtained.

The benzpyrene fraction was evaporated to dryness at 35° *in vacuo* and in darkness. The yield of yellow powdery benzpyrene was 745 mg., 42 per cent, m.p. 168°–171°. This benzpyrene was not sufficiently pure for metabolic studies, and further purification was achieved by additional chromatography.

Because of the sensitivity of benzpyrene to photo-oxidation, the final purification was carried out on 30-mg. batches as often as the pure compound was required for metabolic experiments. Chromatography was again run on a 1×20 cm. column of activated alumina in darkness in 10 per cent benzene in Skelly B as solvent. The solvents were purified by extraction with concentrated sulfuric acid, washing with water, drying over sodium sulfate, and finally by distillation from sodium. The benzpyrene fraction was cut sharply and concentrated *in vacuo* as before. The residue was dissolved in ethanol, precipitated by the addition of water, and was filtered and dried.

The product obtained in 70 per cent yield was crystalline, very faintly yellow in color, m.p. 177°–178°. Its ultraviolet absorption spectrum was identical with that of the purest available sample of benzpyrene, and is considered to be pure enough for use in metabolic studies. The over-all yield based on barium carbonate was 22 per cent. The specific radioactivity obtained by combustion is 2.8 μc. per milligram.

2-Acetylaminofluorene-9-C^{14}

Diphenyl-2-carboxylic acid-C^{14}.—The Grignard reagent was prepared from 0.24 gm. of magnesium, 40 ml. of anhydrous ether, and 2.7 gm. of *o*-iododiphenyl. The reaction started easily, and was complete after heating on the steam bath for 30 minutes. The reaction mixture was titrated and contained 0.34 mM per milliliter.

The carbonation reaction was carried out on the vacuum line as described in "Isotopic Carbon," p. 178 (1). To 10 ml. of anhydrous benzene, placed in the carbonation flask, was added 16 ml. (5.5 mM) of the Grignard reagent solution. The carbonation was run at 0° with 985 mg. (5.0 mM) of barium carbonate containing 5 mc. of radioactivity. After hydrolysis, the product was isolated by evaporation of the solvent, solution in hot N NaOH, and filtration. The filtrate was cooled, acidified, filtered, and the solid was collected and dried. The yield of colorless carboxylic acid was 794 mg. (85 per cent), m.p. 104°–107°. Ray and Geiser (16) reported a yield of 60 per cent. The specific activity was 5.1 μc. per milligram.

Fluorenone-9-C^{14}.—The ring closure was carried out in sulfuric acid solution, but it was found that the yield was considerably improved by the use of 80 per cent instead of concentrated sulfuric acid. To 2.2 ml. of sulfuric acid containing 0.4 ml. of water was added 794 mg. of diphenyl carboxylic acid, the mixture was heated at 85° for ½ hour, and poured onto ice and water. The greenish-yellow product was collected by filtration to give 713 mg. (99 per cent) of fluorenone-9-C^{14} (Ray and Geiser [16], 88 per cent).

Fluorene-9-C^{14}.—A considerable improvement in yield was obtained when the Clemmensen reduction (16) was replaced by the modified Wolff-Kischner reaction (11). A mixture of 713 mg. of fluorenone, 0.51 gm. of KOH pellets, 5 ml. of diethylene glycol, and 0.7 ml. of 85 per cent hydrazine hydrate was refluxed for 3 hours. The solution was a deep purple at first and gradually cleared and became almost colorless. The solution was cooled, poured into water, and the solid was filtered and dried. The yield of slightly darkish fluorene was 598 mg. (92 per cent) (Ray and Geiser [16], 70 per cent).

2-Nitrofluorene-9-C^{14}.—The fluorene was nitrated as described in "Organic Syntheses" (13) and by Ray and Geiser (16). A yield of 523 mg. (71 per

cent), m.p. 156°–158° was obtained (Ray and Geiser [16], 79 per cent).

2-Aminofluorene-9-C^{14}.—The nitro compound was reduced as described in "Organic Syntheses" (10) and by Ray and Geiser (16). The product was crystallized from water, and 410 mg. (94 per cent) of colorless product, m.p. 125°–127° was obtained (Ray and Geiser [16], 90 per cent).

2-Acetylaminofluorene-9-C^{14}.—The acetylation was carried out by the procedure of Ray and Geiser (16) on 355 mg. of aminofluorene, and 403 mg. (97 per cent) of recrystallized product, m.p. 193°–194°, was obtained (Ray and Geiser [16], 84 per cent). Thus, the over-all yield, based on barium carbonate, in this sequence of reactions is 50 per cent. (Ray and Geiser's [16] over-all yield on a larger scale synthesis was 22 per cent.)

The specific activity of the product, as determined by combustion, was 4.55 μc. per milligram. The calculated specific activity of Ray and Geiser's product was 0.30 μc. per milligram.

SUMMARY

The syntheses of two radioactive carcinogens of high specific activity are described in detail.

3,4-Benzpyrene-5-C^{14}, specific activity 2.8 μc. per milligram, was obtained in over-all yield of 22 per cent, based on barium carbonate.

2-Acetylaminofluorene-9-C^{14}, specific activity 4.55 μc. per milligram, was obtained in over-all yield of 50 per cent, based on barium carbonate.

REFERENCES

1. CALVIN, M.; HEIDELBERGER, C.; REID, J. C.; TOLBERT, B. M.; and YANKWICH, P. E. Isotopic Carbon. New York: John Wiley & Sons, 1949.
2. DAUBEN, W. G. The Synthesis of 20-Methylcholanthrene Labeled in the 11-Position with C^{14}. J. Org. Chem., **13**: 313–16, 1948.
3. DAUBEN, W. G. ,and MABEE, D. A Study of the Metabolism of 20-Methylcholanthrene. Cancer Research, **11**:216–20, 1951.
4. FIESER, L. F., and GATES, M. D., JR. Synthetic Experiments Utilizing Perinaphthanone-7. J. Am. Chem. Soc., **62**:2335–41, 1940.
5. FIESER, L. F., and HERSHBERG, E. B. A New Synthesis of 3,4-Benzpyrene Derivatives. J. Am. Chem. Soc., **60**:1658–65, 1938.
6. HEIDELBERGER, C.; BREWER, P. E.; and DAUBEN, W. G. The Synthesis of 1,2,5,6-Dibenzanthracene Labeled in the 9-Position with C^{14}. J. Am. Chem. Soc., **69**:1389–91, 1947.
7. HEIDELBERGER, C., and JONES, H. B. The Distribution of Radioactivity in the Mouse Following Administration of Dibenzanthracene Labeled in the 9 and 10 Positions with Carbon 14, Cancer, **1**:252–60, 1948.
8. HEIDELBERGER, C.; KIRK, M. R.; and PERKINS, M. S. The Metabolic Degradation in the Mouse of Dibenzanthracene Labeled in the 9 and 10 Positions with Carbon 14. Cancer, **1**:261–75, 1948.
9. HEIDELBERGER, C., and WIEST, W. G. The Metabolic Degradation in the Mouse of 1,2,5,6-Dibenzanthracene-9,10-C^{14}. II. 5-Hydroxy-1,2-Naphthalic acid, a New Metabolite. Cancer Research, **11**:511–18, 1951.
10. ———. The Rate of Fecal Elimination of Radioactivity Following a Single Application of 1,2,5,6-Dibenzanthracene-9, 10-C^{14} to the Skin of Mice. *Ibid.*, **11**:255, 1951.
11. HUANG-MINLON. A Simple Modification of the Wolff-Kischner Reduction. J. Am. Chem. Soc., **68**:2487–88, 1946.
12. KINDLER, K., and KWOK, D-Y. Über die Hydrierung von Mandelsäuren und Mandelsäureestern bei katalytischen Hydrierung. Ann., **554**:9–15, 1943.
13. KUHN, W. E. 2-Nitrofluorene and 2-Aminofluorene. Organic Syntheses, Coll. Vol. **2**:447–48, J. Wiley & Sons, New York, 1943.
14. LINSTEAD, R. P., and THOMAS, S. L. S. Dehydrogenation. Part II. The Elimination and Migration of Methyl Groups from Quaternary Carbon Atoms during Catalytic Dehydrogenation. J. Chem. Soc., 1127–34, 1940.
15. MORRIS, H. P.; WEISBURGER, J. H.; and WEISBURGER, E. K. The Distribution of Radioactivity Following the Feeding of Carbon-14 Labeled 2-Acetylaminofluorene to Rats. Cancer Research, **10**:620–24, 1950.
16. RAY, F. E., and GEISER, R. C. Synthesis of 2-Acetylaminofluorene-9-C^{14} and 2-Acetylaminofluorene-ω-C^{14}. Cancer Research, **10**:616–19, 1950.
17. WOLF, G., and HEIDELBERGER, C. 1,2,5,6-Dibenzanthracene-3,4-Quinone and Other Unidentified Quinones as Metabolites of 1,2,5,6-Dibenzanthracene-9,10-C^{14}. Cancer Research, **11**:290, 1951.

Intrinsic Tissue Response to Induction of Pulmonary Tumors*

JOYCE R. SHAPIRO† AND ARTHUR KIRSCHBAUM‡

(Department of Anatomy, School of Medicine, University of Minnesota, Minneapolis 14, Minn.)

The objective of the present investigation was to contribute toward determining the degree to which susceptibility and resistance to carcinogenic action are intrinsic within target tissue, and the extent to which host factors determine tissue reaction. The target tissue investigated was lung; the carcinogen used was urethan.

PRELIMINARY EXPERIMENT

Lung tumors have been induced in several strains of mice by carcinogenic hydrocarbons (1, 2, 11, 17, 20, 21, 25), miscellaneous chemical agents (4, 5, 12), and x-rays (7, 13, 14). By far the most effective method of inducing lung tumors in mice, administration of urethan, was discovered by Nettleship and Henshaw in 1943 (22). In most strains tested for their response to urethan, 100 per cent of the injected mice developed primary pulmonary adenomas, some as early as 3 months after initiation of treatment (6, 9, 10, 23, 24). In all animals the lung was the specific target organ for the carcinogenic action, the incidence of other tumors not being significantly increased.

TABLE 1

INCIDENCE OF URETHAN-INDUCED PULMONARY ADENOMAS IN SEVEN STRAINS OF MICE

Strain	No. of mice	No. with tumors	Per cent	No. of nodules (Range)
Bagg albino	25	25	100	1–18 (10)*
Strong A	23	23	100	5–23 (15)
CBA	25	24	96	1–9 (4)
NH	23	23	100	2–15 (7)
DBA	55	9	16	1–4 (2)
FB	25	3	12	1–3 (1)
FA	28	0	0	0
Bagg×DBA-F_1	33	33	100	5–20 (8)

* Numbers in parentheses represent number of nodules most frequently observed in the strain.

Procedure.—Twenty-five mice from each of several strains, CBA, Bagg albino, Strong A, DBA, FB, FA, and NH, were tested with urethan to determine their relative susceptibility and resistance to pulmonary carcinogenesis. It has been reported that a direct correlation exists between the incidence of spontaneous and carcinogenic hydrocarbon-induced lung tumors—that is, strains with a high rather than a low spontaneous incidence are more susceptible to the carcinogenic induction of lung tumors (3, 11, 15–18, 25). In this preliminary experiment, seven strains of mice were tested to determine whether a similar correlation exists with urethan as the inciting agent.

Anesthetic doses of urethan (1 mg/gm body weight in 10 per cent aqueous solution) were administered intraperitoneally once a week for 6 weeks, starting when the animals were 70 days of age. Mice of each strain were examined at autopsy at intervals of 6, 7, 8, and 9 months after the beginning of treatment. Lungs were removed and examined for nodules visible to the naked eye. Nodules were counted, samples fixed in Zenker's fixative, and paraffin sections were made for microscopic verification of gross diagnosis. Where no nodules appeared grossly, lungs were sectioned at several levels, although not serially.

Results.—With the exception of the NH strain of mice, positive correlation was obtained between the spontaneous, carcinogenic hydrocarbon and urethan-induced incidences of lung tumors. Of the seven strains of mice treated with urethan, four were highly susceptible to its lung tumor-inciting action (Table 1). In the order of their response they were the Strong A (100 per cent incidence, average of fifteen nodules), the Bagg albino (100 per cent incidence, average of ten nodules), the NH (100 per cent, average of seven nodules), and the CBA (96 per cent, average of four nodules). Of these four strains, only the NH has a very low spontaneous incidence, and induced lung tumors in 100 per cent of the injected animals might not have been anticipated. Methylcholanthrene has also been reported to increase the incidence of lung tumors in the Strong A (8, 25),

* This investigation has been aided by a grant from the National Cancer Institute.

† Submitted in partial fulfillment of the requirements for the degree of Master of Science at the University of Minnesota.

‡ Present address: Department of Anatomy, College of Medicine, University of Illinois, Chicago 12, Ill.

Received for publication May 4, 1951.

Bagg albino (19), and CBA strains,[1] the NH not having been tested for its response.

Three strains were refractory to the induction of lung tumors with urethan. The DBA was relatively resistant (16 per cent incidence), as it is to methylcholanthrene (7 per cent incidence). The spontaneous incidence was also very low. The FA and FB strains (genetically related to one another and to the F strain of mice) might be considered a special group, since their response to urethan appears to be inflammatory rather than neoplastic (Fig. 9). Their spontaneous incidence was also very low; response to methylcholanthrene has not been determined.

MATERIALS AND METHODS

Preliminary experiments revealed that the DBA was a good choice for a strain resistant and the Bagg albino for a strain susceptible to the carcinogenic action of urethan.

Bagg albino mice (100 per cent incidence of urethan-induced lung tumors) were mated to DBA mice (16 per cent incidence) to obtain F_1 hybrid offspring. Approximately one-half of the experimental animals were from Bagg albino male×DBA female matings and the others from the reciprocal cross. Hybrids injected with urethan responded as did the Bagg albino parent—that is, 100 per cent developed lung tumors (Table 1).

When the hybrids reached 70 days of age, pulmonary tissue was grafted into their ears. Lung tissue from 1-day-old Bagg albino litters was grafted into the left ear of the hybrid, and similar tissue from DBA litters was grafted into the right ears of the same animals. Small subcutaneous pockets were made into the host's ear (by employing iridectomy scissors and fine forceps), into which the small pieces of lung tissue were forced. The most successful grafts were made with a piece of tissue about 2 mm. in diameter. With larger pieces necrosis of the ear resulted. Within 1 week the incision was healed, and within 2 weeks the grafts were well vascularized.

Fifty-nine mice were operated upon. One week after the grafting was completed, 27 mice taken at random were placed on experiment, while the remainder were kept as controls. Experimental animals were injected with a solution of 10 per cent urethan (1 mg/gm body weight) once a week for 6 weeks. All mice were killed 8–13 months later. All ear grafts were fixed in Zenker's solution and sectioned serially, unless the presence of a tumor in the graft was obvious, in which case sections were made only to confirm the gross diagnosis.

[1] A. Kirschbaum, unpublished data.

RESULTS

Bagg albino tissue residing in injected hosts.—It was generally noted that the Bagg albino lung tissue grew more rapidly and attained a larger size than did DBA lung tissue residing in the same host. Of 27 Bagg albino transplants, 8 regressed within 1 month and were excluded from the data. Two hybrids died 5 months after the experimental procedure (grafting and injection) had been completed and were not available for autopsy.

Nine of the seventeen ear grafts were obviously tumorous (Table 2). In some cases nodules 1–2 mm. in diameter were visible under the skin of the ear. In one instance the tumor had attained a size of 5 mm. (Fig. 1). In some grafts the presence of a tumor was not apparent until the ear had been removed and the graft dissected out. In three additional instances adenomas were demonstrated by examination of serial sections of the grafts. Thus, urethan induced pulmonary adenomas in 76 per cent of the susceptible Bagg albino lung tissue grafts present within a susceptible host.

TABLE 2

INCIDENCE OF ADENOMAS INDUCED BY URETHAN IN BAGG ALBINO LUNG TISSUE GRAFTED TO EARS OF HYBRIDS

Mouse no.	Months in ear	Gross diagnosis	Microscopic diagnosis
254	13	−	−
255	13	−	++
256	13	++	++
252	12	−	−
243	11	−	0*
294	11	++	++
306	11	−	−
296	10	−	−
356	10	++	++
358	10	++	++
295	9	−	++
297	9	++	++
299	9	++	++
354	9	−	++
355	9	++	++
357	9	++	++
258	8	++	++
Total number of mice: 17		Total number with tumors: 12	

* Death from pneumonia, no sections obtained.

Microscopically, the adenomas were well developed and identical histologically with those found in the lungs *in situ* of animals injected with urethan (compare Figs. 2 and 3, 4 and 5). Some areas contained phagocytes with hemosiderin in association with accumulations of lymphocytes (Fig. 8). In some cases the tissue resembled atelectatic lung.

DBA tissue residing in injected hosts.—The right ear of the F_1 hybrids contained the DBA lung tissue. Of 27 DBA grafts, 8 regressed and

were excluded from the data. The 8 failures of both the Bagg albino and DBA tissues were not, however, found in the same hybrid host. At the termination of the experiment the DBA grafts studied were in general smaller than the Bagg albino. All grafts less than 1 mm. in diameter were excluded from microscopic study. None was observed grossly to contain tumors (Table 3). Microscopically, however, it was noted that one graft had a small well formed adenoma (Fig. 7). The grafted DBA tissue resembled the Bagg albino tissue histologically in all respects, except for the lower incidence of induced lung tumors. An incidence of only 6 per cent (one out of seventeen cases) was obtained in DBA lung tissue residing in a host susceptible to the lung tumor-inciting action of urethan.

TABLE 3

INCIDENCE OF ADENOMAS INDUCED BY URETHAN IN DBA LUNG TISSUE GRAFTED TO EARS

Mouse no.	Months in ear	Gross diagnosis	Microscopic diagnosis
255	13	–	–
256	13	–	–
245	13	–	–
246	13	–	–
247	13	–	–
252	12	–	–
243	11	–	0*
294	11	–	–
306	11	–	–
296	10	–	–
356	10	–	–
358	10	–	–
297	9	–	++
354	9	–	–
355	9	–	–
357	9	–	–
258	8	–	–
Total number of mice: 17		Total number with tumors: 1	

* Death from pneumonia, no sections obtained.

Ear-grafted animals not treated with urethan.—Thirty-two animals were set aside with ear grafts but were given no treatment with urethan. Five animals were excluded because of premature death. Of the remaining animals, 25 retained viable Bagg albino lung tissue grafts during the entire experimental period of observation; 16 DBA grafts remained viable. Animals were killed 11–13 months after the grafts were transplanted; serial sections of the grafts were made. One Bagg albino graft had a tumor which might have been induced by transplantation per se but which probably represents a spontaneous occurrence (1 per cent incidence of spontaneous lung tumors in Bagg albinos before 400 days of age). The grafted tissue was well maintained and identifiable histologically as lung (Figs. 6 and 8).

DISCUSSION

The ear as a site for transplantation of lung tissue proved very favorable. The majority of transplants became established readily, grew, and were histologically pulmonary in character (Figs. 6 and 8). The hybrid host was highly susceptible to the lung tumor-inciting action of urethan and supported the growth of tissue from the two parent stocks. Urethan diffuses throughout the body, assuring adequate exposure of the grafted tissue to carcinogenic action. The 76 per cent incidence of lung tumors obtained in the Bagg albino lung tissue indicates a high susceptibility of the grafts to the carcinogenic action of urethan, especially when one considers the relatively small amount of tissue acted upon. It seems logical to suppose that, were it possible to have grafted the entire lung into the ear, a 100 per cent incidence would have been observed. DBA tissue grafted into the ears of the same hosts reacted to urethan as does tissue of this genetic type in its natural environment—that is, within a genetically resistant DBA host.

It can be concluded that the intrinsic capacity of pulmonary tissue to respond to the carcinogenic action of urethan was manifest in tissue grafts. DBA pulmonary tissue maintained its resistance, although residing in a susceptible host during exposure to the carcinogenic chemical. Pulmonary tumors were induced in susceptible Bagg albino grafts under identical conditions.

SUMMARY

Bagg albino (susceptible to the induction of pulmonary tumors by urethan) and DBA (resistant) mice were mated to obtain F_1 hybrids (susceptible). Lung tissue from 1-day-old mice of each of the parent strains was transplanted to the left and right ears, respectively, of the hybrids. Hosts were then injected with urethan. Although the two types of lung tissue grafts were in genetically identical hosts, twelve out of seventeen grafted Bagg albino lungs became tumorous, whereas only one out of seventeen DBA grafts exhibited a tumor. It has been determined, therefore, that susceptibility and resistance to the carcinogenic action of urethan on the lung are intrinsic properties of the target pulmonary tissue and that the host does not determine this reaction.

REFERENCES

1. ANDERVONT, H. B. Pulmonary Tumors in Mice. I. The Susceptibility of the Lungs of Albino Mice to the Carcinogenic Action of 1,2,5,6-Dibenzanthracene. Pub. Health Rep., 52:212–21, 1937.
2. ———. Pulmonary Tumors in Mice. VI. Time of Appearance of Tumors Induced in Strain A Mice Following

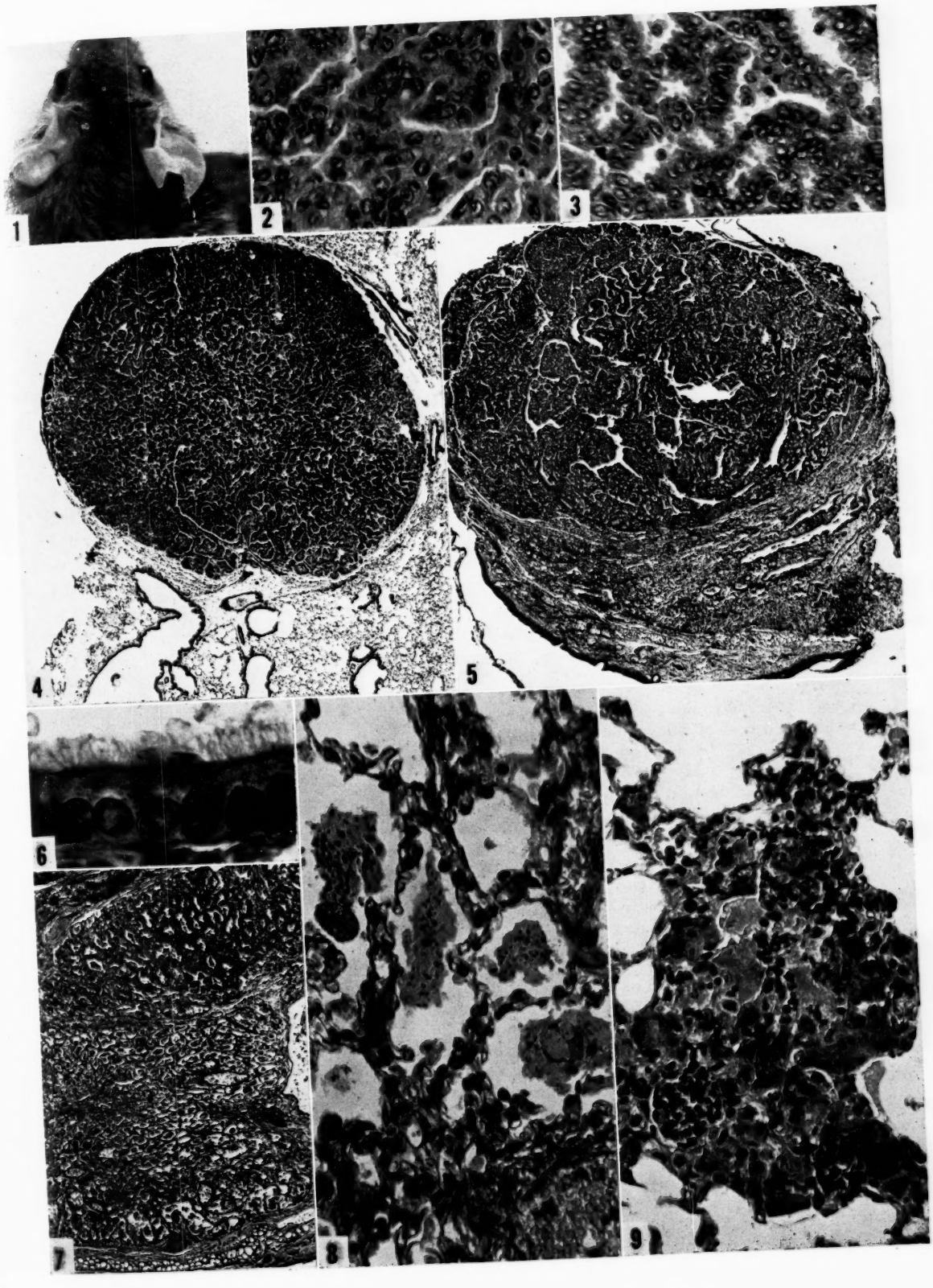

1
2
3
4
5
6
7
8
9

FIG. 1.—Urethan-induced pulmonary adenoma in Bagg albino ear graft (left ear). Tumor, 5 mm. in diameter, exhibited histologic structure illustrated in Figure 3. $\times 1\frac{1}{2}$.

FIG. 2.—Histologic structure of pulmonary adenoma induced by urethan and appearing *in situ*. Composed of closely packed, alveolar-like, cuboidal epithelial whorls with relatively little fibrous stroma. $\times 350$.

FIG. 3.—Section of pulmonary adenoma induced in Bagg albino ear graft. Structure identical with that of urethan-induced adenoma developing *in situ*. $\times 350$.

FIG. 4.—Urethan-induced pulmonary adenoma which developed in lung of Bagg albino $\times$ DBA F_1 hybrid. $\times 45$.

FIG. 5.—Urethan-induced pulmonary adenoma developing in Bagg albino pulmonary tissue graft. $\times 45$.

FIG. 6.—Bronchiolar epithelium within pulmonary tissue graft 13 months after grafting. Section indicates normal appearance of such epithelium. $\times 650$.

FIG. 7.—In upper portion of this section is a circumscribed pulmonary adenoma which developed in a pulmonary graft of DBA tissue residing in a susceptible F_1 hybrid. This is the only instance in seventeen where a tumor developed in resistant tissue within a susceptible host. Histologic appearance of grafted pulmonary tissue is well shown in lower portion of section. Alveoli contain hemosiderin. $\times 45$.

FIG. 8.—Section of pulmonary tissue graft 13 months after grafting. Pulmonary alveoli normal, blood-filled capillaries present in interalveolar septa, hemosiderin in alveoli, phagocytes filled with hemosiderin present. $\times 350$.

FIG. 9.—Inflammatory nodules in lung of FA mouse treated with urethan. Nodules of this type are not neoplastic and are to be distinguished from nodules whose histologic structure is shown in Figures 2 and 3. Leukocytic infiltration and squamous-like cells present. $\times 350$.

Injection of 1:2:5:6-Dibenzanthracene or 20-Methylcholanthrene. *Ibid.*, **54**:1512–19, 1939.

3. ———. Susceptibility of Mice to Spontaneous, Induced, and Transplantable Tumors. A Comparative Study of Eight Strains. *Ibid.*, **53**:1647–65, 1938.
4. ———. Pulmonary Tumors in Mice. IX. The Induction of Pulmonary Tumors in Strain A Mice by Injection of 2-Amino-5-azotoluene or 3:4:5:6-Dibenzcarbozole. *Ibid.*, **54**:1529–33, 1939.
5. BOYLAND, E., and BRUES, A. M. The Carcinogenic Action of Dibenzcarbozoles. Proc. Roy. Soc. London, s.B., **122**: 429–41, 1937.
6. COWEN, P. N. Some Studies on the Action of Urethane on Mice. Brit. J. Cancer, **1**:401–5, 1947.
7. FURTH, J., and FURTH, O. B. Neoplastic Disease Produced in Mice by X-Rays. Am. J. Cancer, **28**:54–65, 1936.
8. GRADY, H. G., and STEWART, H. L. Histogenesis of Induced Pulmonary Tumors in Strain A Mice. Am. J. Path., **16**:417–32, 1940.
9. HENSHAW, P. S., and MEYER, H. L. Minimal Number of Anesthetic Treatments with Urethane Required To Induce Pulmonary Tumors. J. Nat. Cancer Inst., **4**:532–35, 1944.
10. ———. Further Studies on Urethane-induced Pulmonary Tumors. *Ibid.*, **5**:415–17, 1945.
11. HESTON, W. E. Lung Tumors and Heredity. I. The Susceptibility of Four Inbred Strains of Mice and Their Hybrids to Pulmonary Tumors Induced by Subcutaneous Injections. J. Nat. Cancer Inst., **1**:105–11, 1940.
12. ———. Induction of Pulmonary Tumors in Strain A Mice with Methyl-Bis (β-Chloroethyl)amine Hydrochloride. *Ibid.*, **10**:125–30, 1949.
13. LORENZ, E. Radioactivity and Lung Tumors. J. Nat. Cancer Inst., **5**:1–15, 1944.
14. LORENZ, E.; HESTON, W. E.; DERINGER, M. K.; and ESCHENBRENNER, A. B. Increase in Incidence of Lung Tumors in Strain A Mice Following Long-continued Irradiation with Gamma Rays. J. Nat. Cancer Inst., **6**:349–53, 1946.
15. LYNCH, C. J. Studies on the Relation between Tumor Susceptibility and Heredity. III. Spontaneous Tumors of the Lung of Mice. J. Exper. Med., **43**:339–55, 1926.
16. ———. Studies on the Relation between Tumor Susceptibility and Heredity. IV. The Inheritance of Susceptibility to Tar-induced Tumors in the Lungs of Mice. *Ibid.*, **46**: 917–33, 1927.
17. ———. Studies on the Relation between Tumor Susceptibility and Heredity. V. The Influence of Heredity upon the Incidence of Lung Tumors in Mice. *Ibid.*, **54**:747–60, 1931.
18. ———. Strain Differences in Susceptibility to Tar-induced Skin Tumors in Mice. Proc. Soc. Exper. Biol. & Med., **31**: 215–17, 1933.
19. ———. Susceptibility of Mouse Strains to Lung Tumor and Sarcoma Induced by 1,2,5,6-Dibenzanthracene. *Ibid.*, **33**:401–3, 1935.
20. MAGNUS, H. A. The Experimental Production of Malignant Papillomata of the Lung in Mice with 1:2:5:6-Dibenzanthracene. J. Path. & Bact., **49**:21–31, 1939.
21. MURPHY, J. B., and STURM, E. Primary Lung Tumors in Mice Following the Cutaneous Application of Coal Tar. J. Exper. Med., **42**:693–700, 1925.
22. NETTLESHIP, A., and HENSHAW, P. S. The Induction of Lung Tumors in Mice with Ethyl Carbamate (Urethane). J. Nat. Cancer Inst., **4**:309–19, 1943.
23. ORR, J. W. The Histology and Histogenesis of Pulmonary Adenomata of Mice. Brit. J. Cancer, **1**:316–22, 1947.
24. SELBIE, F. R., and THACKRAY, A. C. Lung Adenomas Induced by Urethane in CBA mice. Brit. J. Cancer, **2**:380–85, 1948.
25. SHIMKIN, M. B. Induced Pulmonary Tumors in Mice. I. Susceptibility of Seven Strains of Mice to the Action of Intravenously Injected Methylcholanthrene. Arch. Path., **29**:229–38, 1940.

Studies on the Mechanisms of Metastasis. The Distribution of Tumors in Various Organs in Relation to the Distribution of Arterial Emboli*

DALE R. COMAN, ROBERT P. deLONG, AND MORTON McCUTCHEON

(Department of Pathology, University of Pennsylvania School of Medicine, Philadelphia, Pa.)

The experiments reported in this paper were designed to answer the question: Why are secondary tumors common in some organs and rare in others? Metastatic tumors in man, in Willis' series (8), are most frequent in lungs, liver, bones, kidneys, adrenals, in that order, with spleen and muscle near the bottom of the list.

Two explanations have been advanced to account for the different frequencies with which various organs are sites of secondary tumors: one proposes that the differences in frequency depend on local chemical conditions in the several organs that render them either more or less suitable for maintaining tumor growth. This explanation is known as the "soil" hypothesis. The second hypothesis explains the distribution of metastases as being due to mechanical factors such as blood supply—that is, the chance of tumor emboli lodging in a certain organ.

This latter hypothesis, which is tested in the present experiments, may be stated as follows: the number of metastatic tumors in an organ is proportional to the number of tumor emboli reaching and lodging in that organ. If experimental results are consistent with this explanation, the "soil" hypothesis would be rendered unnecessary.

As regards the predominance of lungs, liver, and bones, i.e., skeletal axis, as sites of metastasis, there can be little doubt that a satisfactory explanation is offered by the mechanical hypothesis. The greatest number of tumor emboli, as they are transported by systemic veins or lymph ducts, lodge in the lungs. Somewhat fewer but still very numerous emboli are transported to the liver, and, as reported by the present writers, great numbers of tumor emboli may be carried by the paravertebral venous system to the bones of the vertebral column, shoulder-girdle, and pelvis. Thus, the areas into which the principal veins drain receive the greatest number of emboli and therefore have the greatest number of secondary tumors, as would be predicted by the mechanical hypothesis.

It remains to consider the arteries as a pathway for dissemination. The arteries carry emboli originating in primary or secondary tumors of the lungs and also emboli that pass through the lungs. Most of the organs, such as kidneys, muscle, spleen, adrenals, receive their emboli through the arteries, yet the resulting metastases vary greatly in number: kidneys and adrenals are frequent sites of secondary tumors, but these are comparatively rare in spleen, muscle, and thyroid. If it could be shown that far more embolic tumor cells lodge in kidneys and adrenals than in spleen, muscle, and thyroid, the mechanical theory would be strongly supported. Obviously, the necessary data cannot be obtained from human material but can be acquired from experimental animals as described below.

MATERIALS AND METHODS

The tumor selected for this investigation was the Brown-Pearce rabbit tumor. In the first part of the experiment, a suspension of cells was prepared by forcing tumor tissue through a sieve. In order to recognize these cells in tissue sections, they were fixed in formalin and stained with iron hematoxylin. The cells were suspended in salt solution, and 0.8 cc. was injected into the left side of the heart. The animal was sacrificed after 1–3 minutes, and sections were prepared from a number of organs and stained with eosin. Through the microscope the tumor cells appeared dark blue against a pink background. The number of single, stained tumor cells and the number of clumps of stained cells were counted in several sections from each organ, and their distribution, whether in capillaries or arterioles, was noted. The areas of the sections were measured with a planimeter, and

* This work was supported by a grant from the Division of Research Grants and Fellowships of the National Institutes of Health, Public Health Service.

Received for publication May 10, 1951.

the result was expressed as cells and clumps of cells per square centimeter.

In the second part of the experiment, another set of rabbits was similarly injected with suspensions of tumor cells that were living. These animals were sacrificed after 1–3 weeks. At autopsy the organs were examined for grossly visible tumors. Sections were stained with hematoxylin and eosin, and the tumors were counted and expressed as number per square centimeter.

From 20 to 23 animals were used in each series. The organs selected as usually receiving emboli through arteries were adrenals, diaphragm, iris, kidney, masseter muscle, pituitary, spleen, testis, and thyroid gland.

TABLE 1

DISTRIBUTION OF STAINED CELLS COMPARED TO DISTRIBUTION OF TUMORS
(Emboli in Capillaries and Arterioles)

	Stained cells (per sq. cm.)	Tumors (per sq. cm.)
Iris	160.9	Confluent
Pituitary	101.2	Confluent
Adrenal	49.3	117.1
Kidney	40.4	99.1
Spleen	26.3	0.1
Thyroid	25.8	1.1
Testis	13.0	2.6
Diaphragm	8.0	2.7
Masseter	2.6	1.1

When the number of stained cells per sq. centimeter is counted in both capillaries and arterioles, correlation between distribution of stained cells and of tumors in the several organs is fair, but not so many tumors were found in the spleen and thyroid as would be predicted from the number of stained cells in those organs.

According to the mechanical hypothesis, the distribution of tumors should be proportional to the relative number of emboli in the several organs.

RESULTS

From the data thus obtained, the number of tumors per unit area may be compared with the number of emboli per unit area in the several organs.

This comparison is made in Table 1. It may be observed that there are great differences in the number of tumors in the several organs. In iris and pituitary, the tumors were so numerous that they could not be counted, while in spleen, thyroid, and muscle they were very few. When the frequency distribution of tumors is compared with that of stained cells lodging in both capillaries and arterioles, a fair agreement is observed; yet there are discrepancies: fewer tumors occurred in the thyroid and spleen than would be expected from the number of emboli. These discrepancies disappear, however, when only those stained cells are counted that lodged in capillaries (Table 2). The agreement is now excellent.

From these observations it appears evident that embolic tumor cells are more likely to establish themselves and form new tumors if they lodge in capillaries than if they are arrested in arterioles. This probability is strongly supported in the present experiments by microscopic examination of very early tumors. These appear to have arisen almost invariably in capillaries. Thus, in the kidney, young tumors were very numerous and occurred nearly always in the glomeruli, taking their origin from cells in glomerular capillary loops or from the distal end of the afferent arteriole where the arteriole breaks up into capillaries (Figs. 1 and 2). A similar origin of minute tumors, from capillaries or sinusoids rather than from arterioles, could be recognized in all the organs included in this series.

Brown-Pearce tumor cells seem to have difficulty in penetrating the walls of arterioles and forming new tumors. Consequently, such organs as spleen (Fig. 3) and thyroid, in which, presumably for mechanical reasons, embolic cells are mostly arrested in arterioles, are seldom the site of metastatic tumors.

TABLE 2

DISTRIBUTION OF STAINED CELLS COMPARED TO DISTRIBUTION OF TUMORS
(Emboli in Capillaries Only)

	Stained cell (per sq. cm.)	Tumors (per sq. cm.)
Iris	142.0	Confluent
Pituitary	65.4	Confluent
Adrenal	38.8	117.1
Kidney	28.8	99.1
Thyroid	3.0	1.1
Diaphragm	2.0	2.7
Testis	1.7	2.6
Masseter	0.9	1.1
Spleen	0.8	0.1

When the number of stained cells per sq. centimeter is counted in capillaries only, correlation between distribution of stained cells and of tumors in the several organs is excellent.

HISTOLOGIC FEATURES OF TUMORS IN THE VARIOUS ORGANS

Iris.—Tumors arose with almost equal frequency in capillaries of papillary projections and in larger, thin-walled vessels in the body of the iris. The stained cells were found in these same locations.

Pituitary.—Tumors rapidly became confluent and therefore could not be counted. The stained cells were found mostly in sinusoids, and in greater numbers in the posterior than in the anterior lobe.

Adrenal.—Both early metastases and stained cells were found chiefly in the cortex close to the capsule (Figs. 4 and 5). As the tumors grew, they extended toward the medulla. The dead cells did not penetrate the capillaries quite so far toward the medulla as did the living cells.

Kidney.—Tumors arose as numerous minute foci nearly always in the glomeruli, taking origin either from the glomerular capillaries or from the afferent arteriole at its junction with the glomerular capillaries. These tumors filled the glomeruli, and eventually the capsular space surrounding the tufts, before invading the surrounding tubules. The embolic stained cells were found in exactly the same locations, and occurred as single cells and small clusters of from two to four cells. Tumors rarely occurred in the medulla, and the stained cells were never found there.

TABLE 3

DISTRIBUTION OF STAINED CELLS COMPARED TO DISTRIBUTION OF SPONTANEOUS METASTASES

Stained cells Descending order of involvement	Tumors (Pearce and Brown) Per cent involvement	
Kidney	Kidney	50
Adrenal	Adrenal	32
Eye	Eye	16
Diaphragm	Diaphragm	13
Pituitary	Pituitary	11
Spleen	Testis	7
Thyroid	Spleen	3
Testis	Thyroid	3

The organs in the first column are arranged according to the frequency with which stained cells lodged in their capillaries after injection into the left side of the heart. The second column presents the order of frequency of spontaneous metastases reported by Pearce and Brown. There is good agreement between the two columns.

Spleen.—As shown in the tables, almost all the embolic stained cells were found in the arterioles rather than in the capillaries or sinusoids, where very few cells were found. It appears to be significant that the number of metastases in the spleen is small, corresponding to the inability of the cells to escape from the arterioles.

Thyroid.—As in the spleen, few embolic stained cells were found in the capillaries; and, correspondingly, there were few metastases.

Muscle.—The small number of embolic cells corresponds with the known, relatively small flow of blood to muscle.

Comparison between the Distribution of Stained Embolic Cells and Spontaneous Metastases

From these experiments it is concluded that the relative number of tumors arising in various organs depends on the number of embolic cells lodging in the capillaries. If this is true, the distribution of the embolic stained cells should make it possible to predict the distribution of spontaneous metastases. Fortunately, adequate data may be found in the literature detailing the distribution of spontaneous metastases of the Brown-Pearce tumor. In Table 3 the data of Pearce and Brown (6) on the frequency distribution of spontaneous metastases are compared to the frequency that would be predicted on the basis of embolic stained cells in these experiments. To make our data comparable to those of Pearce and Brown, the number of stained cells per square centimeter in the capillary bed of each organ was multiplied by the volume of that organ. It is seen that agreement with Pearce and Brown's data is good. The conclusion therefore seems justifiable that the distribution of metastatic tumors corresponds to the distribution of tumor cell emboli.

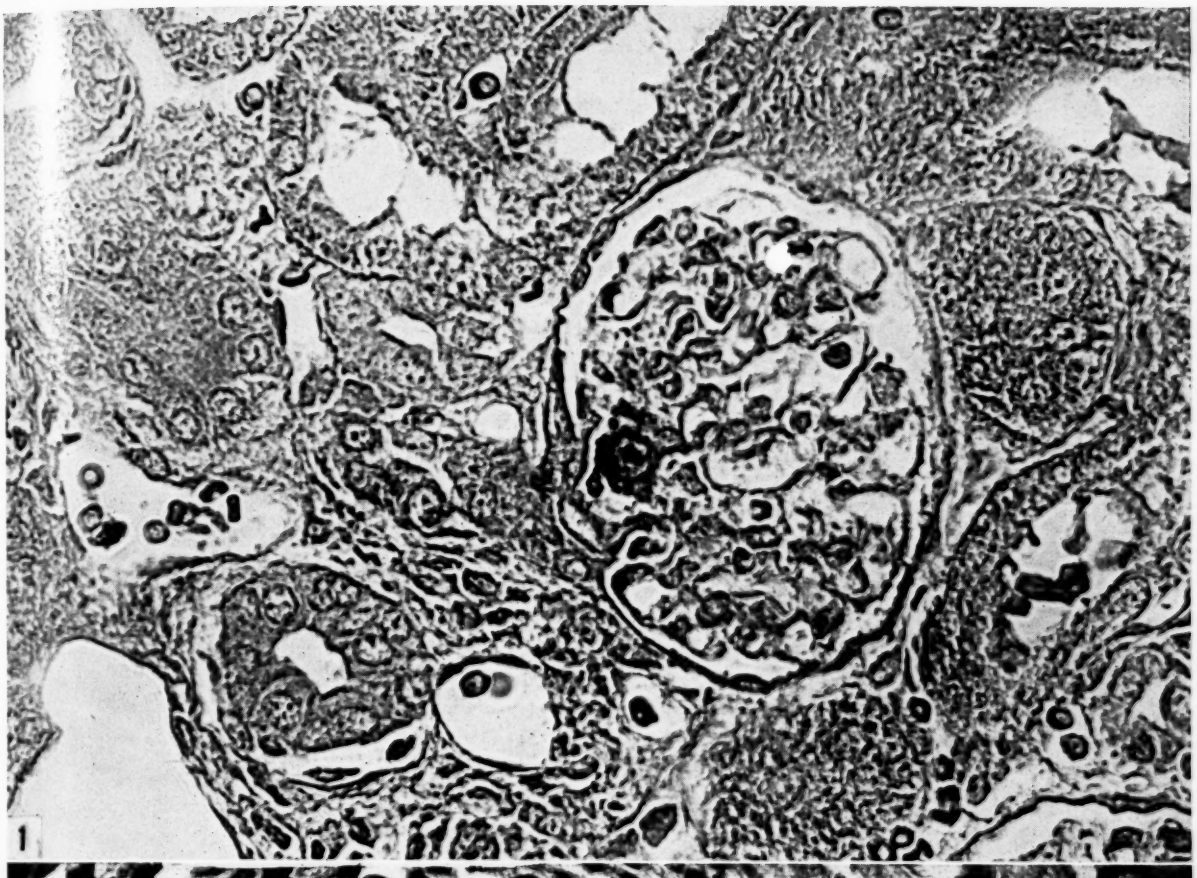
DISCUSSION

In 1889 William Paget (5), in explaining the distribution of metastases in human cancer, likened tumor emboli to "seeds" falling in "soils" of different degrees of fertility. A contrary position was taken by James Ewing (4): "The mechanism of the circulation will doubtless explain most of these peculiarities, for there is as yet no evidence that any one parenchymatous organ is more adapted than others to the growth of embolic tumor cells." Thus, we find stated two hypotheses: the "soil" and the mechanical.

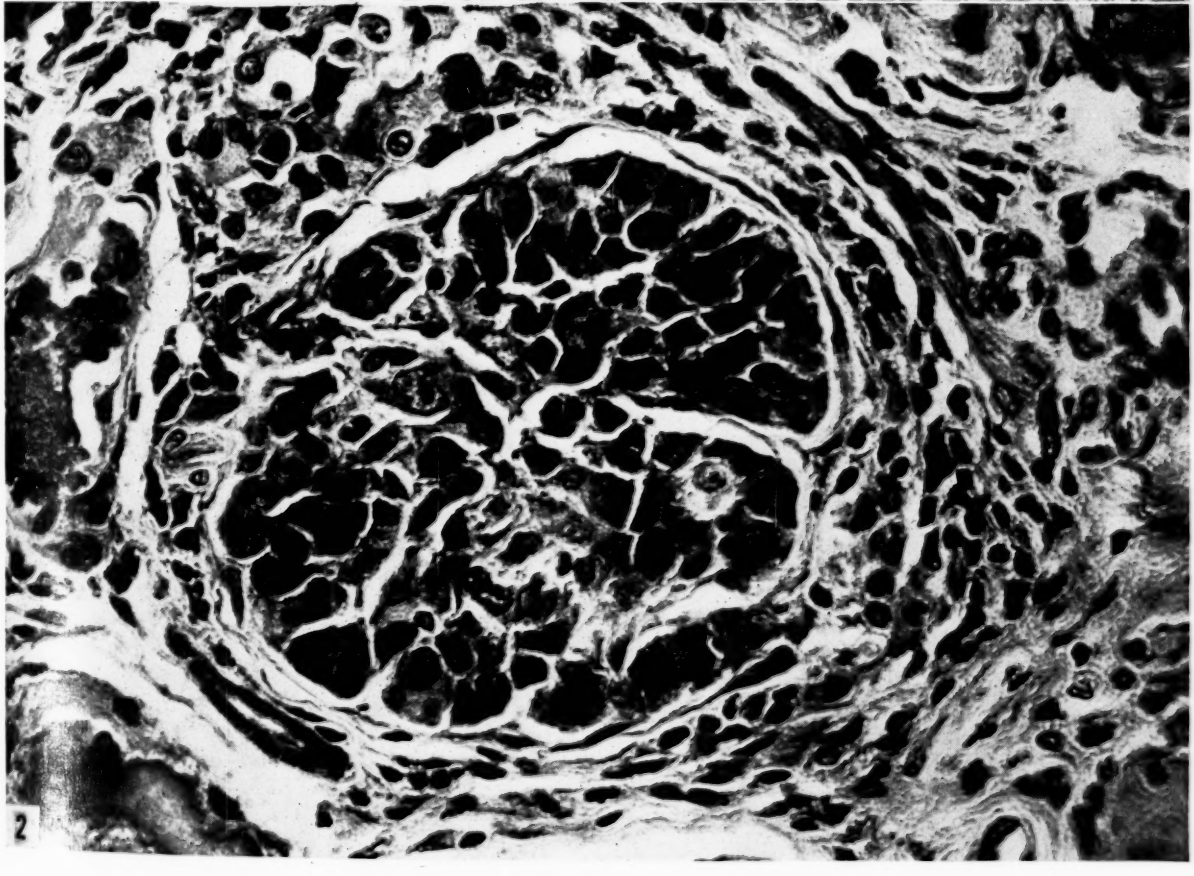
In this and in three preceding papers we have described experiments planned to decide which hypothesis more nearly represents the facts. Necessarily, these investigations have been made upon experimental animals, since adequate and properly controlled data on human cancer are, and will remain, unavailable.

In the first paper (2) the question was asked: Is the low frequency of secondary tumors in some organs due to chemical factors that prevent embolic cells from forming tumors or to the scarcity of embolic cells that reach these organs? The former explanation was not well supported. When cell suspensions of the V_2 rabbit carcinoma, a tumor that rarely produces spontaneous metastases in organs other than the lungs, were injected into the left side of the heart, tumors developed in almost all the organs. It seems likely, therefore, that the rarity of metastases in these organs does not depend upon chemical inhibition but upon the infrequency with which emboli reach these organs.

Next, we attempted to answer the following question: Are metastases common in liver and kidney but rare in spleen and muscle because tumor



1



2

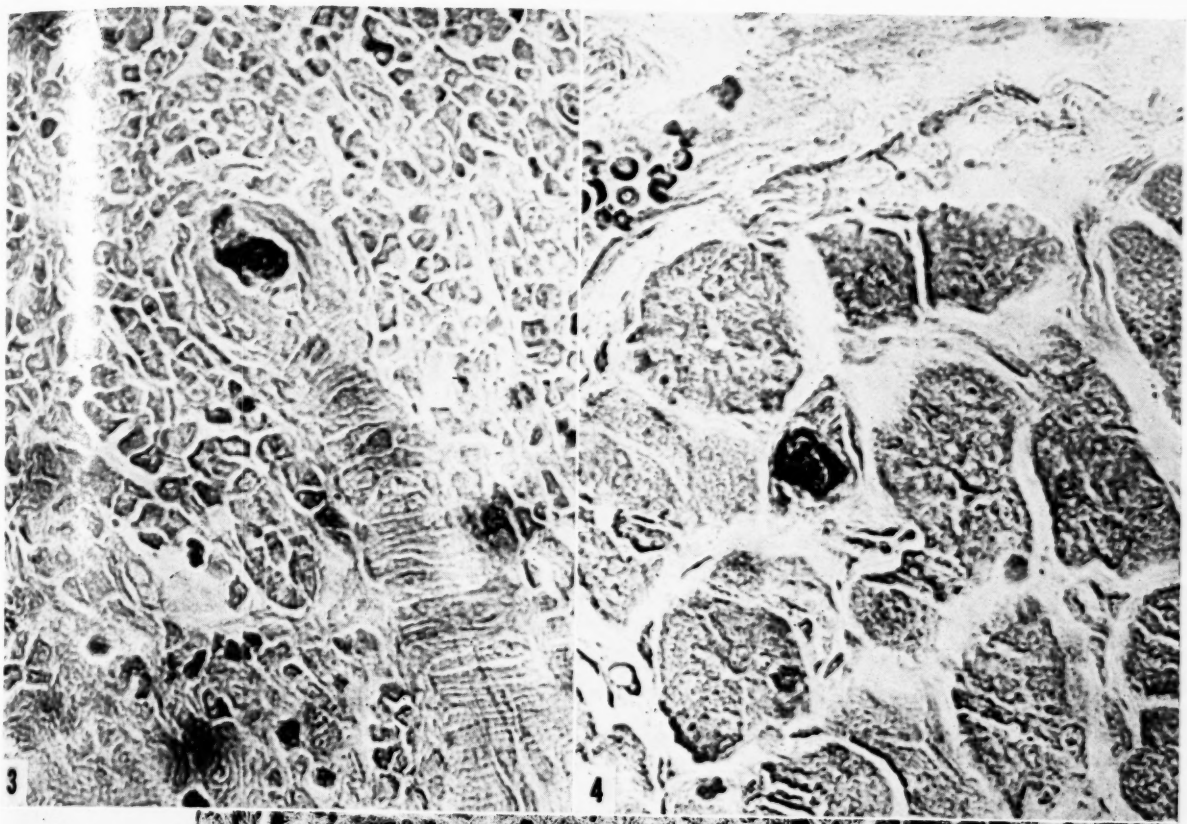
FIG. 1.—Photomicrograph of a renal glomerulus in which a previously fixed and stained cell has lodged in the capillary loops, following injection of a suspension of the cells into the left side of the heart. The deeply stained cell is seen near the center of the photograph adjacent to the entrance of the afferent arteriole. ×600.

FIG. 2.—Photomicrograph of a renal glomerulus that has been largely replaced by proliferating tumor cells. A suspension of viable tumor cells had previously been introduced into the left side of the heart. ×520.

FIG. 3.—Photomicrograph of spleen. In the center is a thick-walled arteriole in which an embolic stained cell has lodged, following injection of a suspension of these cells into the left side of the heart. ×600.

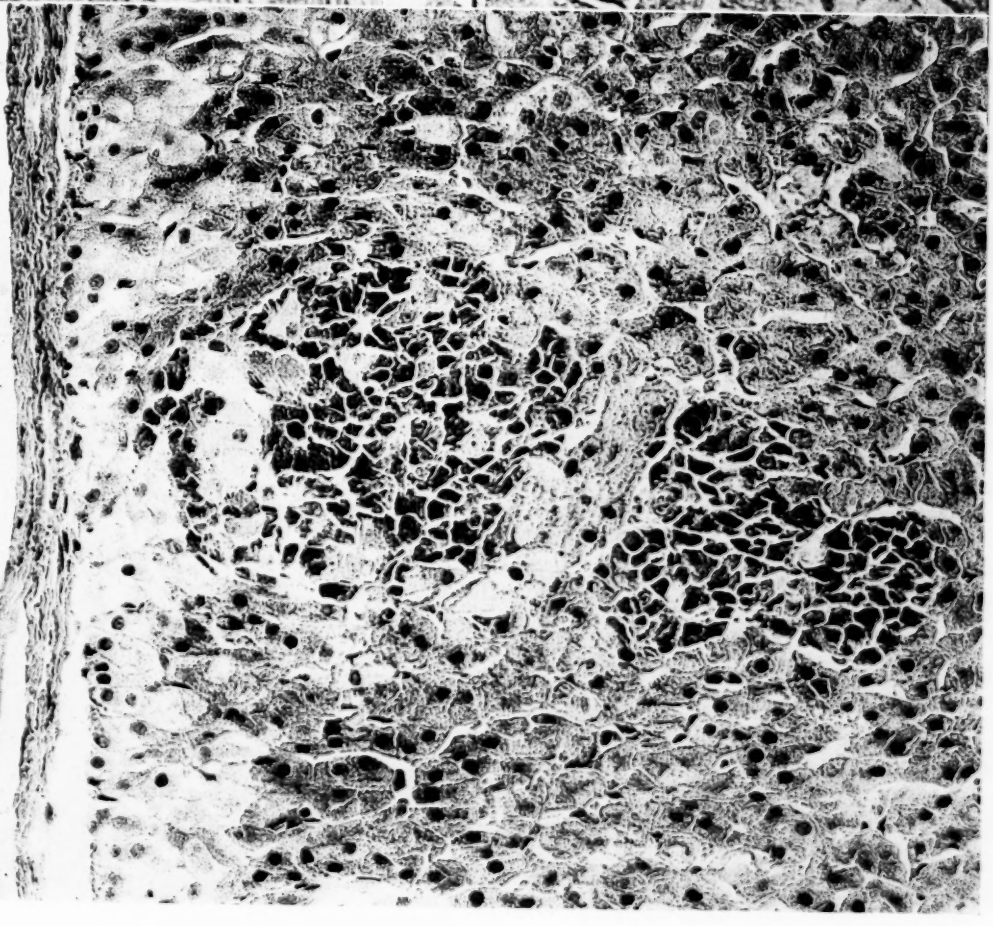
FIG. 4.—Photomicrograph of adrenal gland showing an embolic stained cell lying within a capillary of the cortex. ×600.

FIG. 5.—Photomicrograph of adrenal gland showing tumor nodules in the cortex, as a result of injecting a suspension of viable tumor cells into the left side of the heart. The neoplastic cells are smaller and their cytoplasm stains darker than the adrenal cortical cells. ×300.



3

4



5

cells have difficulty in obtaining a blood supply and stroma and therefore are unable to grow well in spleen and muscle? This explanation (the "soil" hypothesis) was not well supported. When tumor transplants were imbedded in these organs in mice, rats, and rabbits, the tumors obtained a blood supply and grew as rapidly in muscle and spleen as in liver and kidney (3).

It is well known that metastases are common in the vertebral column, shoulder-girdle, pelvis, and skull. Is this high frequency dependent upon peculiarly favorable "soil" conditions or upon the facility with which emboli reach these bones? In a recent paper (1), evidence is presented that great numbers of embolic tumor cells may be carried to these bones by way of the paravertebral veins, in rabbits and in rats, thus acounting for the frequency of secondary tumors.

In the several types of experiments just summarized, no support is found for the "soil" hypothesis. Rather, the inference is that metastasis depends upon how many emboli reach the capillary bed of a given organ.

This conclusion is well supported by the experiments reported in the present paper: where emboli were numerous, tumors were numerous; where emboli failed to reach the capillary bed in good numbers, few tumors grew.

The main support for the chemical explanation of tumor distribution has been the scarcity in man of metastases in such organs as muscles, and their frequency in bones. These peculiarities of tumor distribution have now been satisfactorily accounted for by the relative number of tumor emboli reaching these organs. Species differences in distribution of tumor emboli no doubt exist and presumably account for minor differences in frequency distribution in organs of man and of experimental animals, but the general principle seems well established: the distribution of secondary tumors depends upon the mechanics of circulation, i.e., upon how many emboli reach the capillary bed. "Soil" factors, if they exist at all, must play a minor part.

Of additional interest is the fact that in these experiments tumors arose when the majority of emboli consisted of single cells or small clumps of from two to four cells. This is in contradiction to the assumption that has been presented in the literature (7) that sizable clumps rather than single cells favor the formation of a metastatic tumor. Our results indicate that the embolic cells that are successful in establishing tumors are either single or in clusters tiny enough to penetrate into the capillary bed.

SUMMARY AND CONCLUSIONS

Experiments were designed to determine whether or not the arterial distribution of secondary tumors can be accounted for by the mechanics of circulation and the consequent distribution of embolic tumor cells.

The tumor selected was the Brown-Pearce rabbit tumor. Suspensions of fixed and stained tumor cells were injected into the left side of the heart in 20 rabbits, and the subsequent distribution of the cells was determined by microscopic section. The number of embolic stained cells per square centimeter was calculated for the various organs. In another series of 23 animals, viable cells were similarly injected, and the distribution of resulting tumors was determined.

When the total number of stained cells reaching an organ was compared with the number of tumors in that organ, correspondence was only fair. However, if only those embolic cells that lodged in the capillaries were considered, correspondence was excellent. Thus, the greatest number of emboli per square centimeter was found in iris, pituitary, adrenals, and kidneys; the smallest number in muscle, thyroid, and spleen. The same frequency distribution was found for the tumors.

These results, with the tumor and species studied, are in agreement with the hypothesis that the distribution of metastases is adequately accounted for by the mechanics of circulation and the consequent distribution of embolic tumor cells. In the organs studied, the role played by local chemical "soil" factors is evidently a minor one, if it exists at all.

REFERENCES

1. COMAN, D. R., and DELONG, R. P. The Role of the Vertebral Venous System in the Metastasis of Cancer to the Spinal Column. Experiments with Tumor Cell Suspensions in Rats and Rabbits. Cancer, **4**:610–18, 1951.
2. COMAN, D. R.; EISENBERG, R. B.; and MCCUTCHEON, M. Factors Affecting the Distribution of Tumor Metastases. Experiments with V_2 Carcinoma of Rabbits. Cancer Research, **9**:649–51, 1949.
3. DELONG, R. P., and COMAN, D. R. Relative Susceptibility of Various Organs to Tumor Transplantation. Cancer Research, **10**:513–15, 1950.
4. EWING, J. Neoplastic Diseases, p. 86. 3d ed. Philadelphia & London: W. B. Saunders Co., 1928.
5. PAGET, S. The Distribution of Secondary Growths in Cancer of the Breast. Lancet, **1**:571–73, 1889.
6. PEARCE, L., and BROWN, W. H. Studies Based on a Malignant Tumor of the Rabbit. V. Metastases. Part 1. Description of the Lesions with Especial Reference to Their Occurrence and Distribution. J. Exper. Med., **38**:347–66, 1923.
7. WILLIS, R. A. Pathology of Tumours, pp. 172–74. St. Louis: C. V. Mosby Co., 1948.
8. ———. *Ibid.*, p. 178.

Comments and Communications

With the ever-increasing numbers of manuscripts being submitted for publication, the necessity for brevity and concomitant clarity of papers assumes an ever-increasing importance. There is no objection to the length of papers, provided that the data warrant it; the difficulty is with the flatulent style. Most authors realize the necessity for conciseness, but some are far too wordy, redundant, and repetitious in style. The following quotation was selected from a reviewer's comments concerning a recent manuscript, and it clearly expresses this problem.

"The inclosed treatise is representative of present day doctoral dissertations which are often formidable volumes, and possibly rightly so, since they satisfy the candidate's ego, give evidence of his erudition, and present items for extended philosophical discussion by his examining committee. These considerations, however, have no bearing on publication in scientific journals; the dissertation is rarely, if ever, published *in toto*. *Cancer Research* must maintain a balance by publishing only the essential material which cannot be found elsewhere.

"All authors rebel at the necessity of condensing their material, but it is too often the fact that prolixity conceals rather than reveals. In the present instance, I have had to work very hard to excavate the essential facts from the accompanying *obiter dicta*. I doubt that the reader's interest can be maintained in the face of all this spadework. It is also possible that I have missed pertinent ideas and facts. If so, that is all the more reason for this manuscript to be revised spatially and rhetorically. If the purpose, method, and results cannot be perceived clearly, it is because the paper is not properly written."